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The Humanized Mouse Model: The Study of the Human Alloimmune Response: A Dissertation

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THE HUMANIZED MOUSE MODEL:

THE STUDY OF THE HUMAN ALLOIMMUNE RESPONSE

A Dissertation Presented

By

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ABSTRACT

The transplantation of allogeneic cells and tissues for the treatment of human disease has been a life-saving procedure for many thousands of patients worldwide. However, to date, neither solid organ transplantation nor bone marrow transplantation have reached their full clinical potential. Significant limitations to the advancement of clinical transplantation stem from our current inability to prevent the rejection of allogeneic tissues by the immune system of the host. Similarly, in patients that receive allogeneic bone marrow transplants, we cannot permanently prevent the engrafted immune system from mounting a response against the patient. This problem, termed graft versus host disease is the most prevalent cause of morbidity and mortality in recipients of allogeneic bone marrow transplants.

Clinically, we rely on lifelong immunosuppression to prolong survival of allogeneic tissues within the host. Our currently available therapeutics burden patients with side-effects that range from being unpleasant to life-threatening, while in most cases offering only a temporary solution to the problem of alloimmunity. Efforts are underway to develop protocols and therapeutics that more effectively prevent the pathology associated with alloimmunity. To minimize patient risk, extensive pre-clinical studies in laboratory animals are conducted to predict clinical responses. In the case of immunologic studies, many of these pre-clinical studies are carried out in murine models. Unfortunately, studies of murine immunity often do not predict outcomes in the clinic.

One approach to overcome this limitation is the development of a small animal model of the human immune system.

In this dissertation, we hypothesized that NOD-*scid IL2rγ*^{null} mice engrafted with human peripheral blood mononuclear cells (PBMC), termed the hu-PBMC-NOD-*scid IL2rγ*^{null} model, would provide a model that more accurately reflects human immunity *in vivo* than other models currently available. To investigate this possibility, we first investigated whether NOD-*scid IL2rγ*^{null} mice were able to support the engraftment of human PBMC. We found that NOD-*scid IL2rγ*^{null} mice engraft with human PBMC at much higher levels then the previous gold standard model, the NOD-*scid* mouse. We then investigated the kinetics of human cell engraftment, determined the optimal cell dose, and defined the influence of injection route on engraftment levels. Even at low PBMC input, NOD-*scid IL2rγ*^{null} mice reproducibly support high levels of human PBMC engraftment. In contrast to previous stocks of immunodeficient mice, we observed low intra- and inter-donor variability of engraftment.

We next hypothesized that the human PBMC engrafted in NOD-*scid IL2rγ*^{null} mice were functional and would reject transplanted allogeneic human tissues. To test this, human islets were transplanted into the spleen of chemically diabetic NOD-*scid IL2rγ*^{null} mice with or without intravenous injection of HLA-mismatched human PBMC. In the absence of allogeneic PBMC, the human islets were able to restore and maintain normoglycemia. In contrast, human islet grafts were completely rejected following injection of HLA-mismatched human PBMC as evidenced by return to hyperglycemia and loss of human C-peptide in the circulation. Thus, PBMC engrafted NOD-*scid*

IL2rγ^{null} mice are able to provide an *in vivo* model of a functional human immune system and of human islet allograft rejection.

The enhanced ability of NOD-*scid IL2rγ*^{null} mice to support human cell engraftment gave rise to the possibility of creating a model of graft versus host disease mediated by a human immune system. To investigate this possibility, human PBMC were injected via the tail vein into lightly irradiated NOD-*scid IL2rγ*^{null} mice. We found that in contrast to previous models of GVHD using human PBMC-injected immunodeficient mice, these mice consistently (100%) developed GVHD following injection of as few as $5x10^6$ PBMC, regardless of the PBMC donor used. We then tested the contribution of host MHC in the development of GVHD in this model. As in the human disease, the development of GVHD was highly dependent on host expression of MHC class I and class II molecules.

To begin to evaluate the extent to which the PBMC-engrafted NOD-*scid IL2rγ*^{null} humanized mouse model of GVHD represents the clinical disease, we tested the ability of a therapeutic in clinical trials to modulate GVHD in these mice. In agreement with the clinical experience, we found that interrupting the TNFα signaling cascade with etanercept delayed the onset and severity of disease in this model.

In summary, we conclude that humanized NOD-*scid IL2rγ*^{null} mice represent an important surrogate for investigating *in vivo* mechanisms of both human islet allograft rejection and graft versus host disease.

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Abbreviations

DC, dendritic cell

GVHD, graft versus host disease

G-CSF, Granulocyte stimulating factor

ICAM-1, intercellular adhesion molecule 1

i.p., intraperitoneal

i.s. intrasplenic

i.v., intravenous

LFA-1, Lymphocyte function-associated antigen 1

mAb, monoclonal antibody

MHC, major histocompatibility complex

MST, median survival time

PBMC, peripheral blood mononuclear cell

s.d., standard deviation

TCR, T cell receptor

TNFα, tumor necrosis factor alpha

TBI, Total-body irradiation

CHAPTER I: INTRODUCTION TO TRANSPLANTATION

A Brief History of Transplantation

Transplantation in Antiquity

The idea of transplanting tissues in order to treat a specific condition has a very long history. The Susruta Samhita, an ancient manuscript written by the Indian surgeon Susruta describes a procedure for using a skin flap from the forehead of a patient to repair the deformity caused by amputation of the nose (1). This document, written sometime between 1000 to 500 B.C. by the "Father of Surgery", is the earliest existing manifestation of the love humanity has for the use of surgical techniques to promote medical wellness. Gasparo Tagliacozzi, a 16th century Italian surgeon, adopted Susruta's techniques in rhinoplasty. While he had success with transplanting tissues from one place to another on the same individual, referred to as an autograft, transplanting tissues from one person to another, referred to an as allograft, were repeatedly unsuccessful. In his 1596 work *De Curtorum Chirurgia per Insitionem*, he attributed the failure of allografts to the "force and power of individuality" (2). Over the centuries between the lifetimes of these two scientists' and ours, discoveries in the fields of transplantation and immunology have had monumental impacts on Medicine as it is practiced today.

Benchmarks in transplantation

Since Susruta's time, the investigations into transplanting tissues from one animal to another of the same species (allotransplantation) or from one species to another (xenotransplantation) have resulted in numerous profound discoveries. Many of the scientists responsible for these studies have been awarded a Nobel Prize. Indeed,

transplantation immunology and clinical transplantation have led to more Nobel Prizes than any other field of Medicine (3). Together, the works of these great scientists have been responsible for developing techniques that have saved many thousands of lives.

One of the pioneers in developing surgical techniques that are still used in modern day transplantation was this French surgeon Alexis Carrel (reviewed in 3). Carrel perfected the technique for suturing blood vessels together, without which solid organ transplants (and many other surgical procedures) are not possible. Using the techniques he developed, Carrel and Charles Claude Guthrie carried out a series of experiments in which Carrel observed that a kidney transplanted into a non-biological site in the same animal from which it was harvested would remain functional for the lifetime of the animal (3,4). However, if an organ harvested from one animal was transplanted into another animal of the same species, the organ failed. From this observation, Dr. Carrel concluded that animals had "biological incompatibility", and the idea of an immunologic basis for graft failure was borne (3). Dr. Carrel was awarded the 1912 Nobel Prize in Medicine for his contributions to vascular surgery and transplantation. Despite the integral role he played in the works, Dr. Guthrie was not named in this award, reportedly because of some very controversial head transplants he had attempted (5).

Another pioneer in transplantation was Sir Peter Medawar, sometimes referred to as the "father of transplant immunology". Medawar became interested in ways to improve the outcome of skin grafts during World War II when he was asked to "investigate why it is that skin taken from one human being will not form a permanent graft on the skin of another person" (6). In studies performed in rabbits (7) and humans

(8), Medawar found that skin grafts between two non-identical members of the same species (called homografts or allografts) are rejected. He and his colleagues also recognized that an animal that receives a second graft from the same donor will reject the new graft more quickly (second-set rejection) than the first graft (first-set rejection), a process referred to as the amnestic response (9,10). His results led him to be the first to report that organ rejection was an immunologic phenomenon (7,8,11) For his many contributions to the field of transplantation, Peter Medawar shared the 1960 Nobel Prize in Medicine with Sir Frank Macfarlane Burnet, a major contributor to the clonal selection theory of lymphocytes.

The concept of Tolerance

In his 1960 Nobel Lecture, Peter Medawar stated that "Immunological tolerance may be described as a state of indifference or non-reactivity towards a substance that would normally be expected to excite an immunological response" (6). The observation that led him to this statement began with a report by Ray Owen describing the finding that non-identical twin cattle that shared a placenta would not reject skin grafts from one another, but could still reject skin from other cattle (12). These researchers reasoned that the sharing of blood supply *in utero* resulted in the deletion of lymphocytes that were reactive to the antigens expressed by the fraternal twin through the process of negative selection. Medawar took this observation one step further, hypothesizing that exposure to "non-self" cells early in development would lead to tolerance to the source of those cells. Conversely, exposure to non-self cells later in development would result in sensitization to that antigen. He published this description of immunological tolerance in 1953 (12,13).

The ability to experimentally induce immunological tolerance early in life had been demonstrated as early as 1912. There were reports from the budding field of cancer biology that Rous sarcoma cells could be grown in duck or chicken embryos. The addition of lymphoid cells isolated from adult chickens, in which the sarcoma cells could not grow, caused the sarcoma cells to be destroyed (11). In 1936 Traub made similar observations regarding the persistence of LCMV infection following *in utero* or perinatal inoculation, and the immunological clearance of the same virus in adults (11). The works of Murphy, Traub and Medawar demonstrated that the barriers of transplantation between non-identical subjects could indeed be broken and a state of immune tolerance of donor tissues could be induced.

Immunology of Graft rejection

Discovery of MHC

Dr. Clarence Cook Little, the founder of the Jackson Laboratory in Bar Harbor, Maine, discovered that the "susceptibility and resistance to the rejection of tissues are influenced by multiple genes showing Mendelian inheritance" (14,15). Following up on Little's work, another pioneer in transplant immunology, George Davis Snell, identified the murine histocompatibility locus (H2) as the major genetic determinant of rejection in mouse transplantation models (16). Using congenic mice, animals that differ only at small defined intervals of the genome, Snell discovered each of four mouse strains tested expressed a different allele at the H2 locus. Homologous loci, known as the Major Histocompatibility Complex (MHC) were soon discovered in other species. This work was the basis of his sharing the 1980 Nobel Prize in Physiology or Medicine with Baruj

Benacerraf, who showed that genetic factors related to the H-antigens were responsible for interactions between immune cells, and consequently determined the strength of the immune response, and Jean Dausset who discovered H-antigens in humans (HLA). In his Nobel Lecture, Snell stated

> *Genes competent to play this role in the appropriate experimental or surgical context are called histocompatibility or H genes. An influence on transplants probably is entirely irrelevant to the true function of such genes, but the influence does give the geneticist a handle by which to study them. It was by this route that, over a period of a good many years, I became involved first in immunogenetics and then in the new and fascinating area of cellular immunity (14).*

A series of landmark studies by Doherty and Zinkernagel gave functional significance to Snell's findings. These researchers found that the targeted *in vitro* killing of lymphocytic choriomeningitis virus (LCMV) infected cells by T-lymphocytes required the effector cells and target cells to share expression of at least one H2 locus, called a haplotype (17). This demonstration that there was specificity for not only the peptide antigen but for the context in which it was presented earned Doherty and Zinkernagel the 1996 Nobel Prize in Medicine. This knowledge that T-lymphocytes recognize only peptide complexed with proteins of the H2 region (later called major histocompatability complex, or MHC) began to answer other outstanding questions in the transplantation field.

Alloreactivity

Ten years prior to Doherty and Zinkernagel's discovery of the requirement for peptide to be presented in the context of self-MHC, Darcy Wilson and colleagues published a series of papers describing the outcome of mixed lymphocyte reaction (MLR) studies utilizing cells isolated from different strains of rats. These studies demonstrated that in culture, the "small lymphocytes" from one strain of rat proliferated in response to stimulation with cells from a second, genetically distinct strain of rat (allogeneic) but not in response to stimulation with cells from a genetically identical rat (syngeneic) (18-20). Combined with the elucidation of the biological function of MHC, these studies began our understanding of the mechanism of alloreactivity.

Alloreactive T lymphocytes, or cells reactive against non-self MHC, are thought to be generated through a combination of mechanisms. The first mechanism involves polymorphisms in the MHC locus. There is great genetic diversity within the MHC regions between members of a species, with as much as 5-10% genetic variability between alleles. During thymic development, T lymphocytes bearing T-cell receptors that do not react with self MHC/peptide undergo deletion by neglect, while T lymphocytes bearing T-cell receptors (TCR) that react too strongly with MHC/peptide will be deleted. As the remaining thymocytes by default all react with low affinity to self-MHC/peptide complex, some proportion of these cells will react with allogeneic MHC (21).

The second mechanism thought to contribute to the generation of alloreactive T-cells is the degeneracy of the TCR. The ability of a single TCR to recognize multiple different peptide/MHC complexes, referred to as the degenerate nature of TCR, has been

well documented (22). The consequence of this phenomenon is the higher than expected proportion (estimated from 0.1-10%) of cells that recognize allogeneic MHC (23,24) compared to the 0.001% of T-lymphocytes that react to a given peptide/self-MHC complex (25,26). With the presence of such high numbers of alloreactive lymphocytes it is not surprising that the rejection of allogeneic grafts is so robust.

Mechanisms of Graft Rejection

The rejection of allografts can be mediated by both humoral immunity (hyperacute rejection) and by cellular immunity (acute and chronic rejection). Although each type of rejection occurs by different mechanisms and with different kinetics, all three mechanisms must be averted for the transplant recipient to receive the full benefits of the organ or tissues they are receiving.

By far the fastest form of rejection of allogeneic tissues occurs via the humoral, or hyperacute rejection. The first recognized cause of hyperacute rejection was transplantation across mismatches in the ABO blood group antigens (11,27). These glycoproteins were discovered by Karl Landsteiner in 1902, for which he shared the 1930 Nobel Prize in Physiology or Medicine. The glycoprotein in each group (A, B or O) displays slight differences in the attached sugar moiety. In hyperacute rejection, preformed antibodies exist in the host that recognize the ABO glycoproteins of the mismatched donor. These antibodies are thought to exist due to prior sensitization of the host to the donor tissues or due to crossreacivity with antigens from colonizing bacteria (28). Because the ABO glycoproteins are expressed on endothelial cells, these antibodies will bind to the vasculature of a mismatched organ, causing blood clots that block organ

perfusion. Hyperacute rejection can occur due to antigens other than ABO glycoproteins, as well. For example, a patient who has had multiple blood transfusions may have been sensitized to an HLA molecule shared between prior and current donors. Due to rapid testing techniques routinely performed prior to transplantation today, this method of rejection is not often seen.

The remaining types of cellular rejection, both acute and chronic, are primarily mediated by alloreactive T-lymphocytes. There are two mechanisms involved in cellular rejection. In the first mechanism, the recipient's T cells can recognize the donor MHC directly, aptly named direct antigen presentation. In the second mechanism, donor MHC molecules can be processed by host antigen presenting cells (APC) (29), and the resulting peptides can then be presented by host APC, a process termed indirect antigen presentation. Experiments evaluating the percentage of alloreactive cells that recognize intact allo-MHC directly versus recognizing the processed peptides of the indirect pathway suggest that as high as 90% of allospecific cells are utilizing the direct pathway (30). Both direct and indirect pathways of allorecognition are thought to play a role in the alloimmune response (29).

It has been suggested that acute and chronic rejection are linked to differences between the direct and indirect pathways of allorecognition (31,32). Acute rejection generally begins in the first weeks after transplantation of an allograft. The large proportion of recipient T cells that will react with allogeneic MHC (23,24,30,33) without the requirement for antigen processing makes the direct pathway of alloantigen presentation an attractive possible mechanism for the acute rejection of allogeneic tissues.

Consistent with this hypothesis, it has been shown in some human recipients of allogeneic heart transplants that the numbers of T lymphocytes that can directly recognize the MHC of their donor tissues decreases over time (34). This may be explained by the depletion of donor APC by the recipients immune system over time (31,32). Conversely, indirect presentation of alloantigen would not depend on the presence of donor APCs, as the donor-derived alloantigens are processed and presented by host APC. Thus, the pathway would remain a viable mechanism for allorecognition over long periods of time. This suggests that the indirect pathway of allorecognition is the more important mechanism in chronic rejection of allogeneic tissues, which, in the absence of "tolerance", is still the primary barrier to permanent graft survival. There is some clinical data in support of this hypothesis. Hornick *et al* reported finding a higher frequency of donor T lymphocytes capable of indirect allorecognition in patients with chronic rejection than in those without chronic rejection (35).

Although it is interesting to speculate on the relative contributions of direct vs. indirect antigen presentation in the rejection process, the relationship these two pathways have with acute vs. chronic rejection remains to be fully understood. Regardless, it is clear that both of these mechanisms must be targets for therapeutic intervention if permanent graft survival is to become a reality.

Human transplantation

Background

The technical skills required for human organ transplantation were in place long before the first successful solid organ transplant between humans occurred in 1954 at the

Peter Bent Brigham Hospital in Boston (36). In that landmark case, Joseph Murray led a team of surgeons (including John Merrill, Hartwell Harrison, and David Hume) that removed a kidney from a living donor and transplanted it into the donor's twin brother. It was already known at that time that skin grafts between identical twins were readily accepted (11). According to John Merrill (37), the patient's physician, David M. Miller of the Public Health Service in Boston, suggested the transplant based on this information. This first success provided evidence that the techniques themselves were sound, if only better immunosuppression could be achieved.

Immunosuppression

Soon after Medawar's recognition of rejection as an immunological event, efforts to weaken the immune system to promote graft survival began in earnest. The first immunosuppressant attempted was cortisone. Early studies using a rabbit model of skin graft rejection determined that cortisone successfully prolonged graft survival, albeit for only for a few days (10,38). In 1952 some key observations were made by Cannon *et al* regarding the use of cortisone in neonatal transplantation (11,39). The most important observation was that cortisone increased the success rate of transplanted skin to over 20% without increasing mortality. Further, the grafts remained intact even after the drug was discontinued (11). This groundbreaking observation would be the basis for the search for more effective means of suppressing the immune function; a quest many researchers are still trying to fulfill 50 years later.

Cytoablation

The use of irradiation to deplete lymphocytes as a means to bring about graft survival was described in rabbits by Dempster *et al* in 1950 (40) and in mice by Main and Prehn in 1955 (41). Joseph Murray based his early protocol for kidney transplants between non-identical subjects (42) in part on the work of Main and Prehn. This protocol utilized lethal, or myloablative, irradiation plus bone marrow transplantation, and was implemented in two patients (42). Later protocols utilized sublethal irradiation without the addition of bone marrow transplantation in 10 more patients in renal failure (2). Unfortunately, 11 of 12 total patients enrolled in these studies died of infection within one month (42). The single patient who survived had received a kidney donated from his fraternal twin. Perhaps it was thought that because they were related, it would be easier to induce tolerance, as this patient received a lower dose of irradiation in the absence of a bone marrow transplant, and did remarkably well (11). He experienced two episodes of rejection that were treated successfully with low dose irradiation plus cortisone (3,11). He then survived with normal renal function for the next twenty years. The successful fraternal twin kidney transplant was quickly duplicated in Paris by Hamburger *et al* using the same protocol (43). These two studies were joined shortly thereafter by reported successes in kidney transplant between non-related individuals (44).

The Purine Analogs

The anti-metabolite drugs 6-mercaptopurine (6-MP) and azithrioprine were originally developed as anti-leukemic agents (45). The discovery of azithioprine, along with other drugs, by George Hutchings and Gertrude Elion, earned them the Nobel Prize

in Medicine in 1988. These drugs disrupt the de novo pathway of purine synthesis, which is the primary pathway used by lymphocytes. (46). A series of experiments by Schwartz and Dameshek first demonstrated that these compounds had immunosuppressive properties (47), then, along with Meeker (48), demonstrated 6-MP administration delayed rejection of skin grafts in rabbits (49). Others soon published similar results with purine anti-metabolites in canine models of renal transplantation (50-52). Unfortunately, 95% of the animals treated with 6-MP or azithioprine died within 100 days of receiving renal transplants (11). This poor statistic held true for human studies as well, as the first three renal transplant patients who received 6-MP died (53). A major turning point in the quest for superior immunosuppressive agents occurred in 1963, with the report that the less toxic azathioprine, in conjunction with high dose prednisone, was able to reverse rejection in several kidney transplant recipients (54). Furthermore, many of these patients had developed at least a partial immune tolerance to their grafts, and were able to greatly reduce their immunosuppressive dose (54). Azathioprine and the newer generation of purine biosynthesis inhibitor, mycophenolate mofetil (MMF), are still used clinically today.

Cyclosporine A and Tacrolimus

An extract from the fungi *Clindrocarpon lucidum* and *Trichoderma polysporum* represented a new breakthrough in immunosuppressant drugs for transplantation (11). The mechanism of action of this class of drugs is the inhibition of calcineurin, a key component of the T cell activation signaling pathway. Cyclosporine was found to

suppress immune function in a specific and rapidly reversible way without having the bone marrow depression and toxicity issues of earlier drugs (11).

The efficacy of cyclosporine was rapidly proven in animal models of heart, kidney, pancreas and liver transplantation (55-57). When cyclosporine was used in clinical trials (58,59), several serious side effects were discovered. These include toxicity to the kidney and pancreatic islets as well as a high incidence of B cell lymphoma. (11,60-64). Although the next generation of calcineurin inhibitor, called tacrolimus, succeeded in many clinical cases in which cyclosporine had failed (65-67), the side effects of the new drug were not much improved (68). Despite the side effects, this class of immunosuppressant drugs posed significant improvements over those used previously, and are still in use today.

Inhibitors of mTOR

The mammalian target of rapamycin (mTOR) is an important regulator of lymphocyte progression into the cell cycle following signaling through the IL-2 receptor (69). The mTOR inhibitors, sirolimus (rapamycin) and everolimus, prevent the proliferative response of lymphocytes following stimulation (46), and therefore cripple the adaptive immune response to the allogeneic target. Like the other immunosuppressive agents, adverse effects have been reported, including anemia, leukopenia and lymphoid malignancy (70).

Lymphocyte Specific Immunotherapy

Paul Ehrlich, the recipient of the1908 Nobel Prize for Medicine, coined the term “magic bullet” in reference to a compound that would selectively target a specific

pathogen and destroy it without damaging the host tissue. Efforts to identify the magic bullet that would more selectively target lymphocytes to prevent allograft rejection have been a primary focus in transplantation research for many years. Early attempts were based on findings that the depletion of lymphocytes by drainage of the thoracic duct in rats had an immunosuppressive effect (71). This approach was also attempted clinically (11). However, the need for this expensive and inconvenient therapy for 30 days prior to transplant (72) prevented its wide application in the clinic (73).

The idea of using polyclonal antibodies specific for lymphocytes to prevent cellular immunity was proposed by Metchnikoff in 1899 (11). The utility of this crude mixture, called heterologous antilymphocyte globulin (ALG), for transplantation was shown in a rat skin graft model (74). For clinical studies, horses were immunized with human leukocytes, and the ALG was produced from the serum (11, 75). Although this approach was efficacious when used in conjunction with azathioprine and prednisone, (76), high levels of variability between batches made widespread clinical use at that time problematic.

The discovery of hybridoma technology by Kohler and Milstein (77) brought us one step closer to Ehrlich's magic bullet. This technology spurred the development of the monoclonal antibody (mAb) as a therapeutic. The first clinically used mAb was called OKT3 or Muromonob-CD3 (M-CD3), and was raised against the CD3 determinant of T cells. Administration of OKT3 has had some clinical success (11), as has other T-cell specific mAbs, such as alemtuzumab (anti-CD52) (78), Daclizumab (79) and Basiliximab (80) (anti-CD25α). Efilizumab is a monoclonal antibody reactive against the CD11α

chain of leukocyte-function associated antigen 1 (LFA-1) mAb. In mice, anti-LFA-1 treatment has been successful in promoting long term survival of heart allografts (81), although it is unclear if the mechanism involves blocking T-cell activation, proliferation or migration.

Problems with immunosuppression

To date, although we are arguably much closer to Ehrlich's magic bullet, we have yet to discover a clinical therapeutic that successfully modulates the alloimmune response without leaving the patient at risk for severe infections or the development of cancer (82,83). Indeed, the toxicity, and associated illnesses, resulting from these medications leads to a large percentage of adult patients being non-compliant with their medications (84). Despite the great advances clinical transplantation has enjoyed in the last 50 years, there are still many hurdles to overcome to make permanent acceptance of allogeneic grafts a reality. Considering the cumulative effects of these drugs and the young age of many that are in great need for life-saving transplantation, we undoubtedly need better reagents that will more consistently allow the permanent, immunosuppression-free survival of allografts.

Transplantation of Allogeneic Stem Cells

Background

Like allogeneic organ transplantation, the transplantation of allogeneic hematopoietic stem cells (HSC) is a potential curative therapy for a variety of human diseases, including hereditary diseases of the blood (reviewed in 85), metabolic disorders (reviewed in 86), autoimmune disease (reviewed in 87) and cancer (reviewed in 88,89).

These allogeneic stem cells can come from several sources, including umbilical cord blood or bone marrow. Alternatively, stem cells can be "mobilized" to accumulate in the peripheral blood of donors by administrating the granulocyte stimulating factor (G-CFS) analog, Neupogen (filgrastim). Regardless of the source of the cells, the limitations currently faced in allogeneic stem cell transplantation are substantial. The most concerning limitation currently is the inevitable development of graft versus host disease (GVHD).

Graft Versus Host Disease

Early allogeneic bone marrow engraftment studies showed that irradiated mice infused with allogeneic bone marrow and splenocytes developed a "secondary disease", the symptoms of which were diarrhea, weight loss, skin changes and liver abnormalities (90). In 1963, reports of the first clinically successful bone marrow transplantation describe an unfortunate correlation between the murine studies and clinical findings (91, 11). The patient developed a chronic disease similar to secondary disease described in mice, and committed suicide 2 years after the transplant. Secondary disease, also called secondary syndrome, was later recognized to be the result of the infusion of functional donor lymphocytes into the host. In 1966, Rupert E. Billingham put forth a series of requirements for the development of this "reverse rejection" reaction, termed GVHD (92, 90). First, the graft must contain immunocompetent cells. Second, the recipient must be incapable of rejecting the transplanted cells. Third, the recipient and donor must express different tissue antigens (90). This third component demonstrates the importance of

matching HLA antigens between the donor and recipient in bone marrow transplantation, even more so than in solid organ transplantation (93).

Following allogeneic bone marrow transplantation, the incidence of GVHD is directly related to the degree of MHC mismatch (reviewed in 94,95,96). Clinically, GVHD is also seen in patients who received a transplant of perfectly MHC-matched bone marrow (97). This rejection occurs due to differences in minor histocompatibility antigens between the donor and recipient (98). As many as 40% of patients receiving bone marrow transplants from HLA-identical donors develop acute GVHD in response to antigenic differences that lie outside of the HLA loci (97). Thus, even under optimal conditions, the risks associated with allogeneic stem cell transplantation remain high.

Classifications of Graft versus Host Disease

Clinical GVHD can be classified based on the time of onset after transplant or by histological findings. In both cases, the classifications are described as acute or chronic. For epidemiological purposes, acute GVHD is defined as having an onset within 100 days of transplant, and chronic GVHD begins any time thereafter (98). However, the histopathological changes usually associated with acute GVHD can occur after the 100 day mark. Like the histological changes, clinical symptoms of these two disorders are also distinct from each other. For example, acute GVHD often affects skin, liver and the gastrointestinal tract whereas chronic GVHD can affect a wide range of tissues (98). The diversity in the clinical manifestations of GVHD greatly complicates the investigation into the mechanisms underlying this terrible disease.

Incidence of GVHD

The development of GVHD remains the most common complication of allogeneic stem cell transplantation (99). The development of acute GVHD is the most prevalent cause of morbidity in hematopoietic stem cell recipients, and accounts for up to 40% of the mortality in these patients. Additionally, three months after transplantation of allogeneic HSC up to 50% of surviving patients will be affected by chronic GVHD (100). Clearly, a better understanding of these mechanisms and a reliable therapeutic intervention to prevent GVHD while maintaining the immunological benefits of allogeneic stem cell transplantation are needed in order to maximize the potential of this life-saving procedure (98).

The need for improved experimental models of human alloimmunity

Detailed understanding of complex biologic and pathologic processes requires an *in vivo* system that can be manipulated experimentally. Because of obvious ethical constraints inherent to studies in humans, the creation of animal models that faithfully recapitulate the human immune system are crucial for both the basic scientific and medical research communities. Rodent models of human diseases have greatly expanded our knowledge of disease processes without placing patients at risk for adverse events (101,102). Unfortunately, not all findings in animal studies are successfully translated to clinical interventions for human disease (103-105). Immunological studies for transplantation and autoimmune diseases have proven particularly difficult due in part to differences between murine and human immune systems (106-108).

A small animal model possessing a functional human immune system would aid the research and medical communities in many ways. First, it would circumvent the frustrations associated with failures of translation from the bench to the clinic. Second, a much more detailed in vivo analysis of human immunity would be possible. Third, new therapeutics and protocols for the prevention of diseases mediated by allo- or autoreactive cells could be investigated, with the added ability to investigate mechanisms of action without putting patients at risk. Finally, with the advancement of monoclonal antibodies as therapeutics, it would allow for investigations of human specific reagents in a setting in which detailed mechanistic studies could be performed.

CHAPTER II: HUMANIZED MOUSE MODEL

Background

The term "humanized" mouse is used to describe many experimental systems. Here, it refers to the engraftment of human hematopoietic cells into an immunodeficient mouse. There are variations of this model that differ in both the host mouse strain and in the source of human hematopoietic cells. Each of these models has its advantages and limitations, and the choice of model systems must be made based on the specific parameters the investigator wishes to study. For instance, the introduction of hematopoietic stem cells (HSC) obtained from umbilical cord blood allows studies of the developing human immune system *in vivo*. In contrast, mice engrafted with human PBMC are useful for studying human specific infectious reagents, and the allo- and autoimmune processes *in vivo*.

Rationale

While much has been learned from the study of mouse models of immunology, differences between the murine and human immune system often complicate the direct extrapolation of these data to the clinic. For example, much of our knowledge of type 1 diabetes emanates from studies in the nonobese diabetic, or NOD mouse. Specific reagents developed in the NOD mouse have been put into clinical trials, and have uniformly been found unsuccessful. Because of inconsistencies between the murine and human immune system, a small animal model of the human immune system would be a useful tool to bridge the gap between the bench and the bedside without putting patients

at risk. For this purpose, we have developed a humanized mouse model of the human alloimmune response.

History of humanized mouse models

CB17-scid

The first milestone in the world of humanized mice was the discovery of the *Prkdc*^{scid} mutation (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency) mutation in CB17 mice (109). This mutation introduces a stop codon (110) into a gene encoding the catalytic subunit of a DNA-dependant protein kinase (111-115). The absence of functional *Prkdc* expression results in a failure to activate a DNA recombinase enzyme (116) which, in turn, results in a failure of V(D)J recombination of the lymphocyte antigen receptors (115,117,118) and a general absence of mature, functional B and T lymphocytes (119). As a result, these mice were able to support engraftment with human PBMC (120), fetal liver and thymus (121) and HSC (122). Although the early demonstrations of human cell engraftment were a successful "proof of principal,", the levels of human cell engraftment in these models was extremely low (0.01-0.1%).

Several characteristics of CB17-*scid* mice limited the utility of this model for the study of the function of engrafted human cells. First, despite being homozygous for the *scid* mutation, some adult mice bearing the *scid* mutation developed functional lymphocytes (123). This phenomenon, called "leakiness", affected from 2-23% of young adult mice and as many as 90% of older mice. Second, the defective DNA repair machinery, in addition to disrupting lymphocyte development, also resulted in an

enhanced sensitivity to ionizing radiation (124), which markedly reduces the lifespan of irradiated mice. Third, perhaps in response to the absence of adaptive immunity, CB17-*scid* mice have been shown to have enhanced function of the innate immune system compared to the wild-type strain (125). It was hypothesized that the enhanced innate immune function, and increased natural killer (NK) cell activity in particular (126) remained a barrier to the engraftment of human cells (127).

Efforts to overcome these limitations included the creation of mice lacking recombination activating gene 1 (*Rag1*) (128) or *Rag2* (129) expression. This approach more specifically disrupted lymphocyte development, and did overcome the radiation sensitivity of *scid* mice. However, barriers to human cell engraftment presented by intact innate immune function remained (127).

In an effort to overcome the barrier presented by high levels of innate immunity in CB17-*scid* mice, the scid mutation was introduced onto several different murine backgrounds (131). Mice harboring the *scid* mutation on the NOD background consistently reached levels of human cell engraftment that were 5 to10-fold higher than other strains when injected with PBMC (131) or human spleen cells (132). In 1995, these studies firmly placed the NOD-*scid* model as the "gold standard" for human engraftment studies.

NOD-scid

The NOD mouse is a well characterized model of spontaneous autoimmune diabetes (133). These mice have been reported to have multiple immune defects, including defects in macrophage development and function (134,135) an inability to

activate the hemolytic complement pathways (136), and decreased NK cell activity (133,137). Preventing the development of lymphocytes in the NOD mouse by introducing the *scid* mutation onto this background resulted in an animal that did not develop autoimmune diabetes and retained many of the innate immune defects of the parent strain (125).

The NOD-*scid* mouse is resistant to the leakiness seen in CB17-*scid* mice, as only about 10% of mice have immunoglobulin detectable in their serum at 6 months of age compared to 90% of CB17-*scid* mice at the same age (125). Similarly NOD-*scid* mice are able to retain allogeneic skin grafts over long periods of time, suggesting an absence of functional T-lymphocytes (125).

Levels of engraftment of human PBMC in NOD-*scid* mice are higher than that seen in CB17-*scid* mice (131). Because of this, these mice were used to establish a model of human islet allorejection (138). In this model, mice were rendered diabetic with a dose of streptozotocin, a toxic chemical structurally similar to glucose. Streptozotocin is able to enter insulin-producing β-cells through a glucose transporter. Once inside the β-cell, it causes DNA damage which ultimately renders the animal diabetic. In the NOD-*scid* model of human allorejection, chemically diabetic NOD-*scid* mice were transplanted with a combination of isolated human islets of Langerhans and MHC-disparate human PBMC. Although higher levels of human cell engraftment were attained, only partial rejection of human islets was seen (138).

Despite the improvements that the NOD-*scid* strain presents over previous models, many limitations remained to using these mice as hosts for human cell

engraftment. First, the radiosensitivity conferred by the *scid* mutation would not be improved by the NOD background. Second, NOD-*scid* mice have a mean lifespan of only 8 months due to the development of fatal thymic lymphomas (125,139). Over 70% of NOD-*scid* mice develop thymic lymphomas by 40 weeks of age (125,139), possibly due to an endogenous ecotropic murine leukemia provirus locus, called Emv30, expressed only in the NOD mouse (139). Third, while the engraftment of human PBMC in NOD-*scid* mice is much improved over the CB17-*scid* mouse, the level of human cell engraftment is still relatively low (1-10%) (131). This may be due to the remaining NK cell activity in NOD-*scid* mice (125). In an effort to increase further the engraftment of human cells, methods for genetically blocking the development of NK cells were tested.

Genetic block in NK cell activity

The first of three stains of immunodeficient mice that were developed to genetically disrupt NK cell activity was the NOD-*scid* $\beta 2m^{null}$ mouse (140). As expected, these mice lack NK cell activity and engraft with human PBMC at higher levels than NOD-*scid* mice. However, problems remain with the use of this strain for long term studies of human cell engraftment. First, the lifespan of these mice proved to be even shorter than that seen in NOD-*scid* mice, primarily because the thymic lymphomas that develop in NOD-*scid* mice occur even earlier in life in NOD-*scid* $\beta 2m^{null}$ mice (140). Second, because β2-microblobulin is also involved in regulating iron absorption (141,142), these mice develop severe hemachromatosis (140).

The second immunodeficient mouse strain genetically modified to lack NK cell activity was the NOD $Rag1^{null}$ $Prf1^{null}$ strain (143). As perforin (*Prf1*) is the key mediator

of NK cell killing (144,145), NK cells were able to develop but were not able to function properly (143). As expected, the lack of NK cell function did result in an increased engraftment of human PBMC and HSC. As these mice harbor the *Rag1* mutation rather than the *scid* mutation, they are also relatively radioresistant (143). Although this model represents a step in the right direction, the engraftment levels in these mice remained variable between donors and among recipients of a single donor.

NOD-scid IL2rγ^{null} mouse

The third immunodeficient mouse strain created to genetically disrupt NK cell activity was the NOD-*scid IL2rγ^{null}* mouse. Deficiency of IL2rγ in humans results in X-linked SCID (146). The IL2rγ chain is required for high affinity binding and signaling through IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors (147,148). NOD-*scid IL2rγ^{null}* mice do not develop the thymic lymphomas observed in NOD-*scid* mice. The resulting increase in median lifespan (>89 weeks) compared to that seen in NOD-*scid* mice (39 weeks) makes longer term experiments possible (149). In addition to the absence of mature lymphocytes, dendritic cell maturation defects, and other innate immune deficiencies seen in NOD-*scid* mice (149), NOD-*scid IL2rγ^{null}* mice also completely lack NK cell activity. This is due to the absence of this cell population, as NK cells require signaling through the IL-2 receptor for survival (149). Importantly, we have previously reported that NOD-*scid IL2rγ^{null}* mice are able to support 6-fold higher levels of human PBMC engraftment compared to NOD-*scid* mice (149). It is the PBMC-engrafted NOD-*scid IL2rγ^{null}* mouse that will be the focus of this thesis.

Specific Goals and Thesis Aims

Detailed knowledge of the mechanisms utilized by the human immune system *in vivo* is prerequisite to developing specific immunomodulators for use in clinical transplantation. Studies in the human population that would address these questions are not possible for obvious ethical reasons. Small animal models of the human immune system are an important tool to bridge the gap between laboratory results and clinical science. Currently available small animal models of human immunity have only partially reflected clinical findings. We hypothesized that the human PBMC engrafted NOD-*scid IL2rγ*^{null} mice, termed the hu-PBMC-NOD-*scid IL2rγ*^{null} model, would be superior to previous models in its ability to accurately engraft with a functional human immune system *in vivo*. The goal of this thesis project was to investigate if a murine model of human immune function based on the NOD-*scid IL2rγ*^{null} mouse engrafted with PBMC could more accurately represent alloimmunity as it exists clinically. To achieve this goal, we devised the following specific aims:

Aim 1: Test the hypothesis that a functional human immune system can be engrafted in NOD-*scid IL2rγ*^{null} mice. Chapter IV presents data investigating this hypothesis. Four goals were addressed: 1) Optimize the parameters for human PBMC engraftment.

2) Investigate kinetics of human PBMC engraftment 3) Evaluate the function of engrafted human PBMC by allogeneic challenge. 4) Begin the *in vivo* studies of human therapeutics in PBMC-engrafted NOD-*scid IL2rγ*^{null} mice.

Aim 2: Test the hypothesis that the enhanced engraftment of PBMC in NOD-*scid* *IL2rγ*^{null} mice will allow a xenograft model of GVHD that accurately mimics clinical disease. Chapter V presents data investigating this hypothesis. Three goals were addressed: 1) Define the parameters of GVHD development in the human PBMC engrafted NOD-*scid IL2rγ*^{null} mice. 2) Investigate the role of host MHC class I and class II in the development of GVHD in human PBMC engrafted NOD-*scid IL2rγ*^{null} mice. 3) Evaluate the modulation of GVHD in human PBMC engrafted NOD-*scid IL2rγ*^{null} mice using clinically relevant human therapeutics.

CHAPTER III: MATERIAL AND METHODS

Animals

NOD/LtSz-Prkdc^{scid} (NOD-scid) and NOD.cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (abbreviated as NOD-scid IL2rγ^{null}) NOD-scid IL2rγ^{null} β2m^{null}, NOD-scid IL2rγ^{null} Ab^{o}, NOD-scid β2m^{null}, NOD-scid Ab^{o} and NOD-scid β2m^{null} Ab^{o} mice were obtained from colonies developed and maintained by LDS at The Jackson Laboratory (Bar Harbor, ME). All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, lactate dehydrogenase-elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, LCMV, polyoma, Mycoplasma pulmonis, and Encephalitozoon cuniculi. They were housed in a specific pathogen-free facility in microisolator cages, given autoclaved food and an alternating schedule of acidified water and a solution of sulfamethoxazole-trimethoprim (Goldline Laboratories, Ft. Lauderdale, FL) (140). Animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences).

GVHD Protocol

NOD-*scid IL2rγ^{null}* mice were irradiated with 2 Gy unless otherwise indicated and injected intravenously 4 hrs later with various doses of PBMC. In all experiments, mice were weighed 2 to 3 times weekly and the appearance of GVHD-like symptoms

including rapid weight loss (>15%), hunched posture, ruffled fur, reduced mobility, and tachypnea were used to determine time of euthanasia and is indicated as time of survival.

Antibodies and Flow Cytometry

FITC conjugated anti-human CD8 (clone HIT8a), PE conjugated anti-human CD4 (clone RPA-T4), PerCP conjugated anti-mouse Ly5 (clone 30-F11), APC-conjugated anti-human CD45 (clone H130), Alexa 700 conjugated anti-human CD3 (clone UCHT1), and anti-mouse CD16/32 (clone 2.4G2) mAb were obtained from BD Pharmingen (San Jose, CA). Alexa 405 conjugated anti-human CD20 (clone 2H7) was obtained from AbD-Serotec (Oxford, UK).

At times indicated in the text, single-cell suspensions of spleens or bone marrow flushed from the femurs and tibias were prepared in RPMI 1640 and washed in complete PBS (Dulbecco's PBS containing 0.1% sodium azide [Sigma-Aldrich, St. Louis, MO] and 1% fetal bovine serum [HyClone, Logan, Utah]). Blood was collected from the retro-orbital venous plexus of anesthetized mice using EDTA-coated capillary tubes (Drummond Scientific, Broomall, PA) and EDTA-treated 1.5 ml tubes (Eppendorf, Westbury, NY). Blood, single cell suspensions of splenocytes, or bone marrow were incubated in anti-CD16/32 for 5 min at 4°C to block nonspecific Fc binding. Antibodies at the appropriate dilutions, previously determined by titration against human or mouse lymphocytes, were incubated with 10^6 spleen cells or 100μl of blood for 30 minutes at 4°C. Spleen cells and blood were then incubated with anti-mouse CD45 and fluorescence-conjugated anti-human CD45 mAb plus anti-human CD3, anti-human CD4, anti-human CD8, and/or anti-human CD20 mAb. Labeled spleen cells were washed, fixed with 1% paraformaldehyde

(Polysciences, Warrington, PA) in complete PBS. Blood samples were processed with FACS lysing solution (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. At least 20,000 events were acquired with an LSRII instrument (BD Biosciences, San José, CA) and analyzed using FlowJo Software (Mac version 8.2; Tree Star, Ashland, OR).

For all analyses, anti-mouse CD45 staining was performed to exclude murine host cells from analysis, and only human $CD45^+$ cells that were mouse CD45-negative were included in the analyses 150. Matching isotype antibodies were used as negative controls and the reported values have been corrected using these isotype control values (143). Levels of human $CD45^+$ cells reaching 0.1% in the blood and 1.0% in the spleen at four weeks were considered as successfully engrafted mice (131,132,140,143).

Collection of human PBMC

Human PBMCs were obtained from healthy volunteers and blood donors, under signed informed consent in accordance with the Declaration of Helsinki and approval from the Institutional Review Board of the University of Massachusetts Medical School. PBMCs were collected in heparin and purified by ficoll hypaque density centrifugation. PBMCs were then suspended in RPMI for injection into mice at the doses and routes indicated.

Quantification of Human Immunoglobulin

Total human IgG and IgM in serum were quantified by ELISA as previously described (140). Briefly, 96 well plates were coated with goat-anti-human IgG $F(ab')_2$ or donkey-anti-human IgM $F(ab')_2$ (Jackson ImmunoResearch, West Grove, PA). A mixture of biotinylated mouse-anti-human Ig lambda (clone JDC-12) and mouse-anti-human

IgKappa (clone G20-193; BD Pharmingen) was added, and streptavidin-alkaline phosphatase (Southern Biotech, Birmingham, AL) with 4-nitrophenyl phosphate (Sigma-Aldrich) was used for detection. Total human IgG and IgM (Jackson ImmunoResearch) standard curves were run with each assay and Ig levels were determined from the curves. Optical densities of ELISA samples were read at 405 nm on a Maxline Emax plate reader (Molecular Devices, Sunnyvale, CA) according to the manufacturers recommendations.

Human Cytokine Production

NOD-*scid IL2rγ*^{null}mice received 2 Gy total body irradiation using a Gammacell 40 (Atomic Energy of Canada, Ottawa, Canada) four hours prior to receiving intravenous injections via the tail vein of 20x10^6 human PBMC. Plasma samples were collected from the retro-orbital venous plexus of anesthetized mice 1 hour, 4 hours and 24 hours after PBMC injection using EDTA-coated capillary tubes (Drummond Scientific, Broomall, PA) and EDTA-treated 1.5 ml tubes (Eppendorf, Westbury, NY). Plasma samples were stored at -80 until use. The Cytometric Bead Array Human Inflammation kit (BD Biosciences, San Jose, CA) was used following manufacturers recommendations to determine the concentration of human cytokines in the plasma samples.

Mixed Lymphocyte Culture

Murine stimulator cells were prepared by preparing single cell suspensions of spleens recovered from NOD-*scid*, NOD-*scid-β2m*^{null}, NOD-*scid Ab*^{o}, or NOD-*scid β2m*^{null} *Ab*^{o} mice. RBC were lysed with hypotonic ammonium chloride lysing solution, and the nucleated cells were washed in RPMI supplemented with 10% FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.29 mg/ml L-glutamine (complete medium); counted;

and suspended in complete medium at $10x10^6$ cells/ml. The stimulator cells were then exposed to 20 Gy ^{137}Cs radiation using a Gammacell 40 (Atomic Energy of Canada). Murine stimulator cells ($5x10^6$ in 500µl) were added to each well of 24-well tissue culture plate (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). Responder human PBMC were labeled with 1µm CFSE (Molecular Probes, Eugene, OR, 151)). Labeled responder cells ($1x10^6$ in 500µl of medium) was added to triplicate wells containing murine stimulator cells, and the plates were incubated for 7 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were then harvested, stained for cell surface markers and analyzed with an LSRII instrument (BD Biosciences). Mod-Fit software (Verity, Topsham, ME) was used to determine precursor frequency.

Human Islet Transplantation

The procurement and use of all human tissues were performed under protocols approved by the Institutional Review Board of the University of Massachusetts Medical School. Human islets designated for research were obtained from the Juvenile Diabetes Research Foundation Islet Isolation Center at the University of Pittsburgh (Pittsburgh, PA) or the National Islet Cell Resource Center Program (NCRR). All islets were maintained in CMRL 1066 culture medium (Media Tech. Inc., Herndon, VA) containing 2.5% human albumin at 25°C in an atmosphere of 95% air 5% CO_2 until transplanted. Islet viability, assessed by trypan blue dye exclusion, was 65–75% and islet cell purity, as determined using dithizone staining, was 60–75%. The islet yield was converted mathematically to a total number of islets with an average diameter of 150 µm [islet equivalents (IEQs)]. Human IEQs (3000–4000) suspended in RPMI were transplanted into spleen of NOD-

scid IL2rγ^{null} mice previously rendered chemically diabetic (blood glucose >450 mg/dl) by a single intraperitoneal (i.p.) injection of streptozotocin (150 mg/kg). The number of islets used for transplantation was determined from preliminary studies showing that 3000–4000 human IEQs routinely reverse hyperglycemia in diabetic NOD-*scid* mice (138). Non-fasting plasma glucose levels were determined using an Accu-Chek Active glucometer (Hoffmann-La Roche, Basel, Switzerland) at twice-weekly intervals following transplantation to monitor islet xenograft function.

Quantification of human insulin and C-reactive peptide

A commercially available assay specific for human insulin and c-peptide and not cross-reactive with mouse insulin or C-peptide was used (Linco Research, St Charles, MO). This assay utilizes differentially labeled fluorescent beads coated with relevant antibody to either insulin or C-peptide and then phycoerythrin labeled secondary antibodies to quantify the hormones using a standard curve. The assay was read using a Luminex analyzer (Luminex, Austin, TX). Manufacturers instructions were followed and the inter- and intra- assay coefficents of variation were 7.2 and 2.6 % for insulin and 4.2 and 7.3% for C-peptide, respectively.

Histopathological analyses

Tissues were recovered from mice at necropsy, fixed in 10% buffered formalin, and embedded in paraffin. Sections 5 μm thick were cut. For routine histology, sections were stained with hematoxylin and eosin. The sections were evaluated by Dr. Bruce Woda or Dr. Oded Foreman without knowledge of the experimental design. For immunohistochemistry, sections were heated at 60°C for 30 min, then deparaffinized and

hydrated through a series of xylene and alcohol baths before staining. The slides were microwaved in a proprietary citrate-buffered Ag retrieval solution (BioTek Solutions, Santa Barbara, CA) for 5 min in an 800-W microwave oven. After replenishment of this solution the slides were microwaved for another 5 min, then cooled for 20 min. Immunohistochemical staining was performed with mAbs specific for human CD45, insulin and glucagon using a DakoCytomation EnVision Dual Link system implemented on a Dako Autostainer Universal Staining System (Dako; Denmark). The sections were counterstained with hematoxylin.

Statistics

All measures of variance are presented as standard error of the mean (SEM). Significance of difference of independent means was assumed for p values of <0.05. Comparisons of two means used the independent samples t-test and comparisons of three or more means used one-way analyses of variance using GraphPad Prism software (Graphpad Software, San Diego, CA).

CHAPTER IV: A NEW HU-PBL MODEL FOR THE STUDY OF HUMAN ISLET ALLOREACTIVITY BASED ON NOD-*SCID* MICE BEARING A TARGETED MUTATION IN THE IL-2 RECEPTOR GAMMA CHAIN GENE

INTRODUCTION

Immunodeficient NOD-*scid* mice bearing a targeted mutation in the IL2 receptor common gamma chain (*IL2rγ*^{null}) readily engraft with human hematopoietic stem cells. Data presented in this chapter describe the analysis of human peripheral blood mononuclear cells (PBMC) as to their ability to engraft in NOD-*scid IL2rγ*^{null} mice. We also describe the kinetics of human cell engraftment, define the optimal cell dose, and investigate the influence of injection route on engraftment levels. Even at low PBMC input, NOD-*scid IL2rγ*^{null} mice reproducibly support high human PBMC engraftment that plateaus within 3-4 weeks. In contrast to previous stocks of immunodeficient mice, we observed low intra- and inter-donor variability of engraftment. NOD-*scid IL2rγ*^{null} mice rendered hyperglycemic by streptozotocin treatment return to normoglycemia following transplantation with human islets. Interestingly, these human islet grafts are rejected following injection of HLA-mismatched human PBMC as evidenced by return to hyperglycemia and loss of human C-peptide. These data suggest that humanized NOD-*scid IL2rγ*^{null} mice may represent an important surrogate for investigating *in vivo* mechanisms of human islet allograft rejection.

RESULTS

1. Engraftment of Human PBMC in NOD-scid and NOD-scid IL2rγ^{null} Mice

NOD-*scid* mice have represented the "gold standard" for engraftment studies involving human lymphohematopoietic cells for over 10 years (127). Several reports have shown that NOD-*scid IL2rγ^{null}* mice support higher levels of engraftment with human hematopoietic stem cells (HSC) than do NOD-*scid* mice (152), but their ability to support engraftment with human PBMC has not been reported. To determine this potential, we compared the engraftment of human PBMC in NOD-*scid IL2rγ^{null}* and NOD-*scid* mice. Cohorts of each strain were injected i.p. or i.v. with 20x10^6 human PBMC and analyzed four weeks later for engraftment of human lymphohematopoietic cells. The i.p. route of injection is the method routinely used for engraftment studies of human PBMC in NOD-*scid* mice whereas few reports have suggested successful human PBMC engraftment in NOD-*scid* mice following i.v. injection (127,132,153,154).

Four weeks after i.p. injection of 20x10^6 PBMC, levels of human CD45$^+$ cell engraftment in the blood of NOD-*scid Il2rγ^{null}* mice (20.7±2.6%, N=26) were significantly higher than those achieved in NOD-*scid* mice (0.1±0.1%, N=11, $p<0.0001$, Figure 1, panel A). Engraftment of human CD45$^+$ cells in the spleen of NOD-*scid IL2rγ^{null}* mice (52.8±4.2%, N=26) was also significantly higher than that achieved using NOD-*scid* mice (1.2±0.8 %, N=11, $p<0.0001$, Figure 1, panel B). Based on these data, all subsequent experiments were performed in NOD-*scid lL2rγ^{null}* mice.

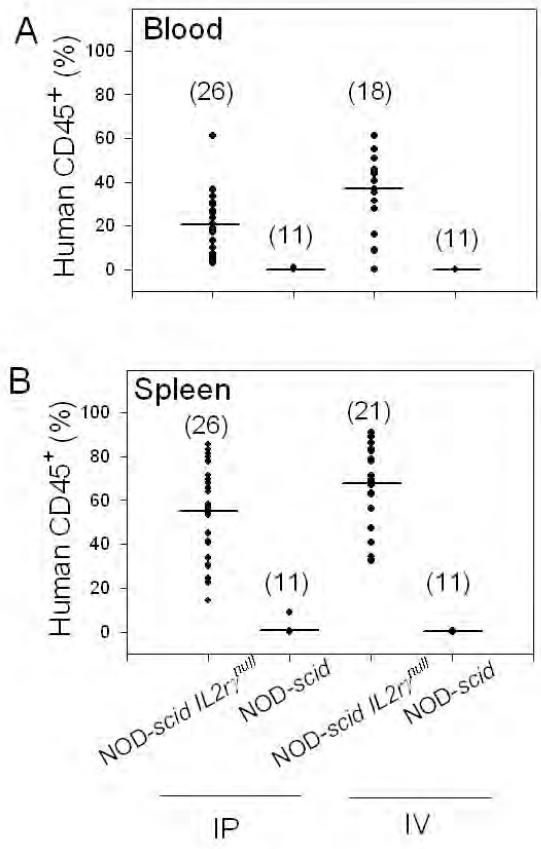


Figure 1. Engraftment of human PBMC into NOD-*scid IL2rγ*^{null} and NOD-*scid* mice. NOD-*scid IL2rγ*^{null} and NOD-*scid* mice were injected with 20x10^6 human PBMC i.v. or i.p. Engraftment of the peripheral blood (Panel A) or spleen (Panel B) was evaluated by flow cytometry 4 weeks later. Bars represent median engraftment levels. (A) NOD-*scid IL2rγ*^{null} mice achieved higher levels of engraftment in the peripheral blood following i.p. ($p<0.0001$) or i.v. ($p<0.0001$) injection. (B) Engraftment of human CD45$^+$ cells in the spleen was higher in NOD-scid lL2rγ^{null} mice than in NOD-scid ($p<0.0001$). Each circle represents an individual mouse.

2. Optimal route of injection for engraftment of human PBMC

We next compared three routes of *in vivo* PBMC delivery that are commonly used; intravenous (i.v.), intraperitoneally (i.p.), and intrasplenic (i.s.) (127,132,138,153,154). Engraftment of human $CD45^+$ cells in the peripheral blood four weeks following i.v. injection of $20x10^6$ human PBMC (37.6±2.7%) was significantly higher than in mice injected either i.p. (20.3±2.8%) or i.s. (22.4±6.4%; $p<0.05$, Figure 2, panel A). As expected, engraftment of human $CD45^+$ cells in the spleen of NOD-*scid IL2r*γ^{null} mice was significantly higher than that achieved in NOD-*scid* mice four weeks after i.v. injection (66.1±4.1% *vs.* 0.1±0.1%, respectively, $p<0.0001$, Figures 1 and 2). The percentages (Figure 2, panel B) and numbers (Figure 2, panel C) of human $CD45^+$ cells engrafted in the spleen four weeks following i.p., i.v., and i.s. injection were statistically similar.

3. Kinetics of Human PBMC Engraftment

To evaluate the kinetics of human cell engraftment, NOD-*scid IL2r*γ^{null} mice were injected i.v. with $20x10^6$ human PBMC. Engraftment of human $CD45^+$ cells in the peripheral blood and spleen was then evaluated weekly over the next four weeks. The percent of human cells increased through three weeks in both blood (Figure 3, panel A) and spleen (Figure 3, panel B). Higher levels of engraftment were observed in the spleen (69.5±6.3%) as compared to that achieved in the blood (37.6±2.7%) at 4 weeks. The number of human $CD45^+$ cells in the spleen also reached a plateau by three weeks following PBMC injection ($4.5±1.7x10^7$, Figure 3, panel C). The majority of the engrafted human cells in the blood and spleen were $CD3^+$ (>98%). The CD4:CD8 ratio of

$CD3^+$ T cells in the blood was variable at week one, but approximated a ratio of ~1:1 at two weeks and remained at this ratio through the four week observation period in both the blood and spleen (Table 1). We next determined the circulating levels of human IgM and IgG. Four weeks after injection of $20x10^6$ human PBMC, high levels of IgM (39.3 ± 18.8 μg/ml, N=5) and IgG (40.9 ± 11.1 μg/ml, N=5) were observed in the serum of engrafted mice, suggesting the engraftment of activated human B cells or xenoactivation of transferred naïve B cells.

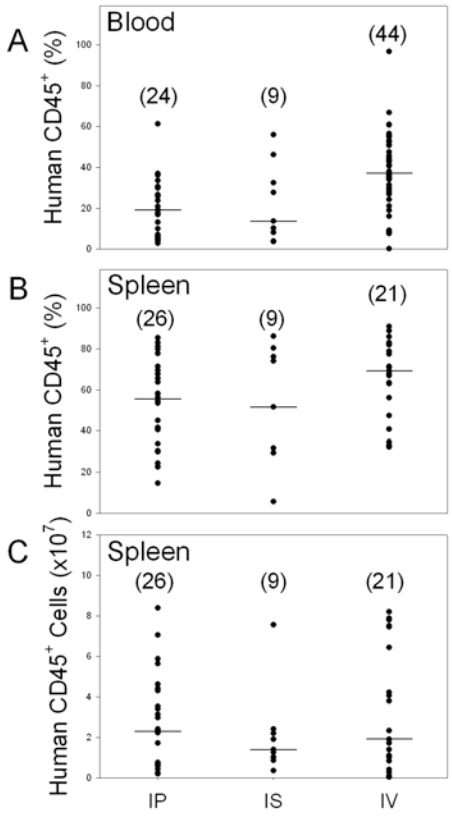


Figure 2. Routes of human PBMC injection.

NOD-*scid IL2rγ*^{null} mice were injected via the i.p., i.s., or i.v. route with 20x10^6 PBMC. Engraftment of human CD45$^+$ cells was evaluated 4 weeks later by flow cytometry. Bars represent median levels of human cell engraftment. Panel A: Intravenous injection of PBMC resulted in significantly higher average percentages of CD45$^+$ in the blood of recipients as compared to either i.p. ($p<0.05$) or i.s. injection ($p<0.05$). Panel B: The percentages of human CD45$^+$ cells engrafted in the spleen were statistically similar for mice injected with PBMC via the i.p., i.s., or i.v. route. Panel C: The average numbers of CD45$^+$ cells recovered from the spleens following i.p., i.s., i.v. injection were statistically similar between all groups. Each circle represents an individual mouse.

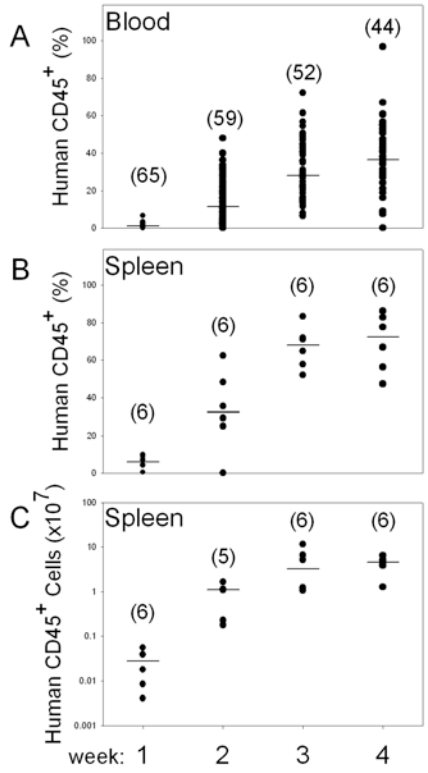


Figure 3. Kinetics of PBMC engraftment.

NOD-*scid IL2rγ*^{null} mice were injected i.v. with 20x10^6 PBMC and evaluated by flow cytometry weekly over a 4 week period. Panel A: The percentage of human CD45$^+$ cell engraftment in the peripheral blood increased significantly each week through 4 weeks ($p<0.01$). Panel B: Engraftment of the percentage of human CD45$^+$ cells in the spleen increased significantly each week through 3 weeks ($p<0.01$), reaching a plateau that did not increase further at week four (p=n.s.). Panel C: The numbers of human cells recovered from the spleens of engrafted mice increased from through week two ($p<0.05$). No further increase in the number of human CD45$^+$ cells in the spleen was achieved at week three or week four (p=n.s.). Each circle represents an individual mouse.

4. Cell dose dependence of human CD45+ cell engraftment

Robust human $CD45^+$ cell engraftment in NOD-*scid* mice following PBMC injection requires i.p. injection of as many as $50x10^6$ to $300x10^6$ human PBMC (127,152,155). For many studies, particularly for experiments using PBMC obtained from children, this represents a significant limitation as fewer cells are typically available. As most published studies have used the i.p. route of PBMC injection, we first determined the engraftment levels of human $CD45^+$ cells in NOD-*scid IL2rγ*^{null} mice following i.p. injection of $20x10^6$ and $50x10^6$ human PBMC. As shown in Figure 4, panels A and B, the percentage of human $CD45^+$ cells engrafted at four weeks was not elevated in recipients of $50x10^6$ as compared to recipients of $20x10^6$ PBMC (p=N.S.).

Having demonstrated that i.v. injection of $20x10^6$ human PBMC led to high levels of human $CD45^+$ cell engraftment (Figures 1-3), we next determined if lower cell doses would lead to human $CD45^+$ cell engraftment in NOD-*scid lL2rγ*^{null} mice. Human $CD45^+$ cells were detected in the blood and spleen of mice four weeks after injection of as few as $1\text{-}5x10^6$ PBMC (Figure 4, panels C and D). Increasing the number of injected PBMC to $10x10^6$ increased the level of engraftment, again with higher levels of engraftment observed in the spleen as compared to the blood (Figure 4, panels C and D). Intravenous injection of $10x10^6$ or more PBMC resulted in engraftment of human $CD45^+$ cells in 100% of the recipients.

Table 1. Engrafted T-lymphocytes develop an activated phenotype

Cell Subset	Day 7	Day 14	Day 21	Day 28
Peripheral Blood				
Total CD4+ (%)	69.3±8.3	45.7±10.0	40.3±13.3	56.1±19.9
% CD45RO+/CD4+	53.9±12.3	89.0±4.9	84.8±5.5	93.9±3.9
% HLA-DR+/CD4+	5.2±2.7	13.0±6.4	50.6±10.3	56.2±23.8
Total CD8+ (%)	30.0±10.3	52.8±10.4	54.1±13.6	42.5±20.6
% CD45RO+/CD8+	63.3±21.9	88.1±6.0	86.3±6.5	93.8±4.5
% HLA-DR+/CD8+	11.9±7.6	22.6±14.5	49.3±6.0	51.2±18.8
CD4:CD8 Ratio	2.3:1	0.9:1	0.7:1	1.3:1
Spleen				
Total CD4+ (%)	20.5±2.9	44.6±6.5	55.5±2.0	51.5±22.3
% CD45RO+/CD4+	77.7±4.7	84.1±0.9	83.8±4.4	95.2±3.6
% HLA-DR+/CD4+	11.4±2.3	4.3±2.0	49.7±10.9	35.7±24.6
Total CD8+ (%)	70.8±6.9	48.8±8.2	43.4±1.6	44.2±20.7
% CD45RO+/CD8+	19.8±14.3	44.4±13.3	94.0±4.1	95.6±2.8
% HLA-DR+/CD8+	62.7±10.1	48.5±10.2	52.7±9.9	48.0±27.9
CD4:CD8 Ratio	0.3:1	0.9:1	1.3:1	1.2:1

Table 1. NOD-*scid IL2rγ*^{null} mice were injected i.v. with 20x10^6 human PBMC and the percentage and phenotype of engrafted human CD3$^+$ cells that were CD4$^+$ or CD8$^+$ in the blood and spleen were analyzed by flow cytometry at the times indicated (n=3).

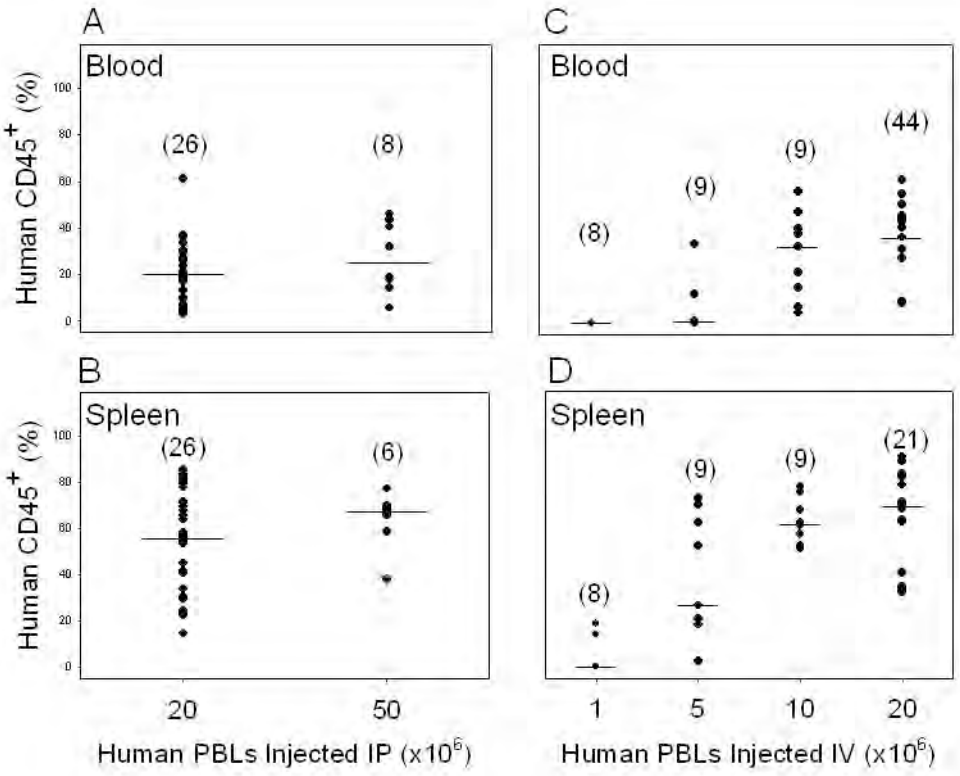

A
Blood
Human CD45+ (%)
(26)
(8)
C
Blood
(8)
(9)
(9)
(44)
B
Spleen
Human CD45+ (%)
(26)
(6)
D
Spleen
(8)
(9)
(9)
(21)
20
50
Human PBLs Injected IP (x10^6)
1
5
10
20
Human PBLs Injected IV (x10^6)

Figure 4

Figure 4. Cell dose dependence of human CD45$^+$ cell engraftment

NOD-*scid IL2rγ*^{null} mice were injected by the indicated route and human PBMC cell dose. The engraftment of human CD45$^+$ cells was determined 4 weeks later. Panels A and B: Mice were injected i.p. with 20x10^6 or 50x10^6 human PBMC. The percentages of human cell engraftment in the peripheral blood (Panel A) and spleen (panel B) are shown. Injection of 20x10^6 or 50x10^6 PBMC resulted in statistically similar levels of human cells in the blood (p=n.s.) or the spleens (p=n.s.). Panels C and D: Mice were injected i.v. with the indicated number of human PBMC. The percentage of human CD45$^+$ cells in the blood (Panel C) increased from undetectable levels (<0.01%) following injection of 1x10^6 PBMC to readily detectable levels following injection of 5x10^6 PBMC. Injection of 10x10^6 ($p<0.025$) or 20x10^6 ($p<0.025$) human PBMC led to increased engraftment. No statistically significant differences were observed in the percentage of human CD45$^+$ cells following injection of 10x10^6 and 20x10^6 human PBMC. Panel D: The percentage of human CD45$^+$ cells in the spleen following injection of 1x10^6 human PBMC increased with increasing cell doses to 10x10^6 human PBMC ($p<0.025$) but was not increased further by injection of 20x10^6 human PBMC (p=n.s.). Each circle represents an individual mouse

5. Intra- and inter-donor variability of human CD45$^+$ cell engraftment

Intra- and inter-donor variability in human CD45$^+$ cell engraftment in recipients of human PBMC has been a major limitation in previous PBMC-engrafted immunodeficient mouse models (152). To assess the variability of human CD45$^+$ cell engraftment in NOD-*scid IL2rγ^{null}* mice, we determined engraftment levels among recipients of single PBMC donors or cohorts of mice given PBMC from different donors. The percentages of human CD45$^+$ cells in the blood and spleen, as well as the number of human CD45$^+$ cells in the spleen and blood, were quantified 4 weeks following i.v. injection of human PBMC into NOD-*scid IL2rγ^{null}* mice.

In contrast to the low frequency of engraftment and the low levels of human PBMC observed in engrafted NOD-*scid* mice (127,131,152), all NOD-*scid IL2rγ^{null}* mice injected with 20x10^6 PBMC from the same donor engrafted with human CD45$^+$ cells (Figure 5, panels A and B). While cells from all donors led to engraftment in NOD-*scid IL2rγ^{null}* mice, the percentage of human CD45$^+$ cells varied considerably, particularly in the peripheral blood. In the spleen, however, the differences between individual mice engrafted with cells from the same donor were small.

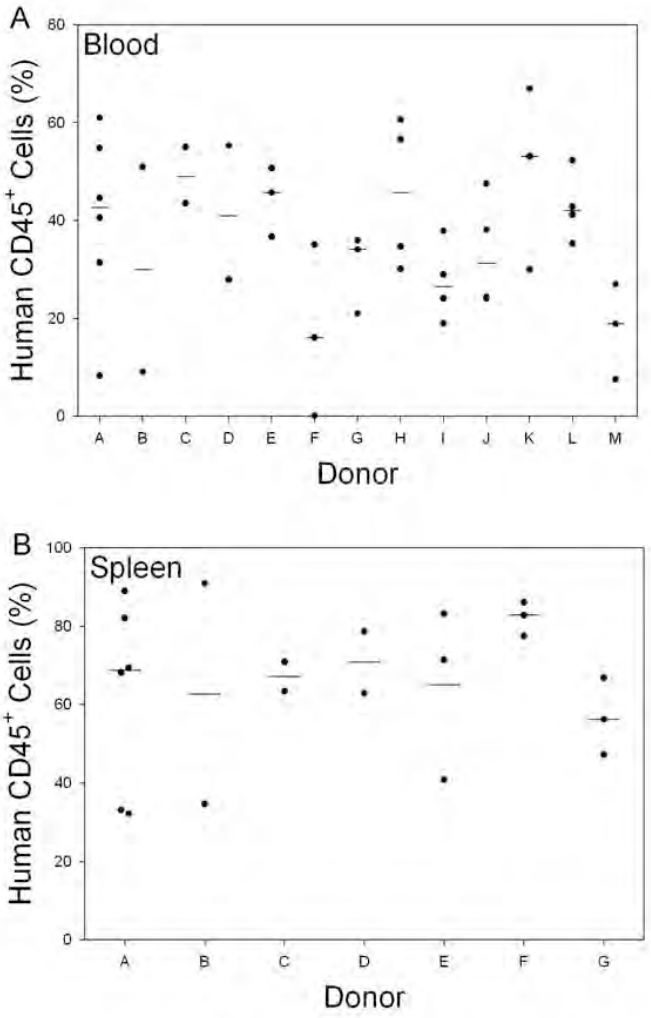


Figure 5. Intra- and inter-donor variability in engraftment among PBMC donors

NOD-*scid IL2rγ*^{null} mice were injected intravenously with 20x10^6 PBMC from seven different donors (x-axis, A-G). Human CD45$^+$ cells in the peripheral blood (Panel A) and spleen (Panel B) were analyzed 4 weeks later. There was no statistical difference in engraftment levels achieved among different PBMC donors in either the blood or spleen. Each circle represents an individual mouse.

6. Engrafted human T-lymphocytes rapidly acquire an activated phenotype

It has previously been reported that the majority of human lymphocytes engrafted into immunodeficient mice acquire an activated/memory phenotype (CD45RO, MHC class II-positive) (150). To investigate the kinetic progression of human $CD3^+$ T cell activation in NOD-*scid IL2rγ*^{null} engrafted mice, splenocytes were isolated from mice at various times after i.v. injection of PBMC and stained for the expression of activation markers. We observed a rapid upregulation of MHC class II on $CD3^+$ T cells and a shift over time to expression of CD45RO, peaking at approximately three weeks (Table 1).

7. Human islet allograft rejection in NOD-scid IL2rγ^{null} mice engrafted with allogeneic human PBMC

We have previously shown that i.p. injection of $300x10^6$ human PBMC into chemically diabetic NOD-*scid* mice transplanted with human allogeneic islets leads to islet graft damage, and in some cases complete rejection (138). To investigate human islet allograft rejection in NOD-*scid IL2rγ*^{null} mice, we first determined whether human islet grafts would restore normoglycemia in chemically diabetic mice transplanted i.s. with human islets. In 14 of 14 diabetic recipients receiving 3000-4000 human islet equivalents, normoglycemia (blood glucose <250 mg/dl) was achieved within five days (Figure 6). Simultaneous i.v. injection of $20x10^6$ allogeneic human PBMC in 100% (8/8) islet graft recipients led to rapid rejection of the islets and a return to hyperglycemia within 21 days. To determine if human PBMC could reject established human islets grafts, normoglycemic mice provided islet transplants 37 days earlier were injected i.v. with $20x10^6$ human PBMC. Two of three mice reverted to hyperglycemia within three weeks

following PBMC injection (Figure 6). Blood glucose levels were increasing in the third mouse prior to termination of the experiment.

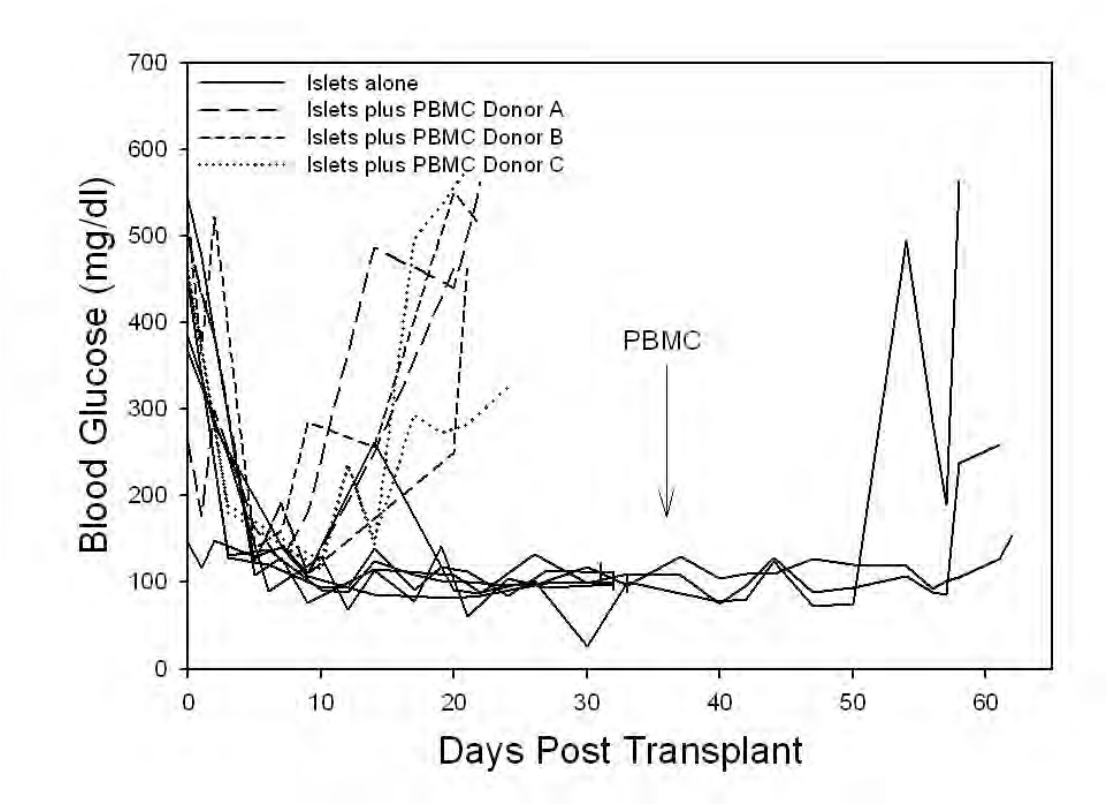


Figure 6. Human islet allograft transplantation in chemically diabetic Hu-PBL-NOD-*scid IL2rγ*^{null} mice.

Chemically diabetic NOD-*scid IL2rγ*^{null} mice were transplanted intrasplenically with 3000-4000 human islet equivalents. Cohorts of these mice also received intravenous injections of HLA-mismatched human PBMC from one of 3 donors on the same day. Blood glucose levels were monitored twice weekly to evaluate the function of the transplanted islets. At day 37, 3 mice bearing functioning human islet grafts that had not received human PBMC were injected intravenously with $20x10^6$ HLA-mismatched PBMC. Vertical bars indicate censored data.

8. Human islet histology

Analysis of human islets transplanted into the spleens of diabetic mice that were not given human PBMC showed intact islets with no islet inflammation 32 days after transplantation (Figure 7, panel A). In contrast, islets in mice injected with human PBMC displayed a marked cellular infiltration around and within the islet grafts (Figure 7, panel B). Staining with anti-human CD45 mAb demonstrated no evidence of human $CD45^+$ cells in mice given islet grafts only (panel C), whereas an intensive human $CD45^+$ cellular infiltrate was observed in the islet grafts of PBMC-injected mice (panel D). Islet grafts in mice not engrafted with human PBMC exhibited intact islets with a normal endocrine cell mass for insulin (panel E) and glucagon (panel G). The spleens of mice given human PBMC showed multiple nodules of lymphoid cells. Some islets in mice given human PBMC were devoid of endocrine cells and some showed a few residual endocrine cells (illustrated in Figure 7, panels F and H).

To confirm human islet function in transplanted mice, human insulin and C-peptide levels were determined in serum obtained 9-12 days (all mice were normoglycemic at this time) and 19-22 days following islet transplantation. In mice given islet transplants only, circulating levels of human insulin and C-peptide were readily detected at both time points (Figure 8). In mice transplanted with human islets and provided allogeneic human PBMC on the day of islet transplantation, high levels of human insulin and C-peptide were detected at days 9-12. In contrast, by days 19-22, time points at which hyperglycemia had returned in human PBMC injected mice, only low levels of human insulin or C-peptide were detected in the serum (Figure 8), consistent

with the high blood glucose levels and morphological evidence suggesting that the human islets had been rejected by the engrafted human PBMC.

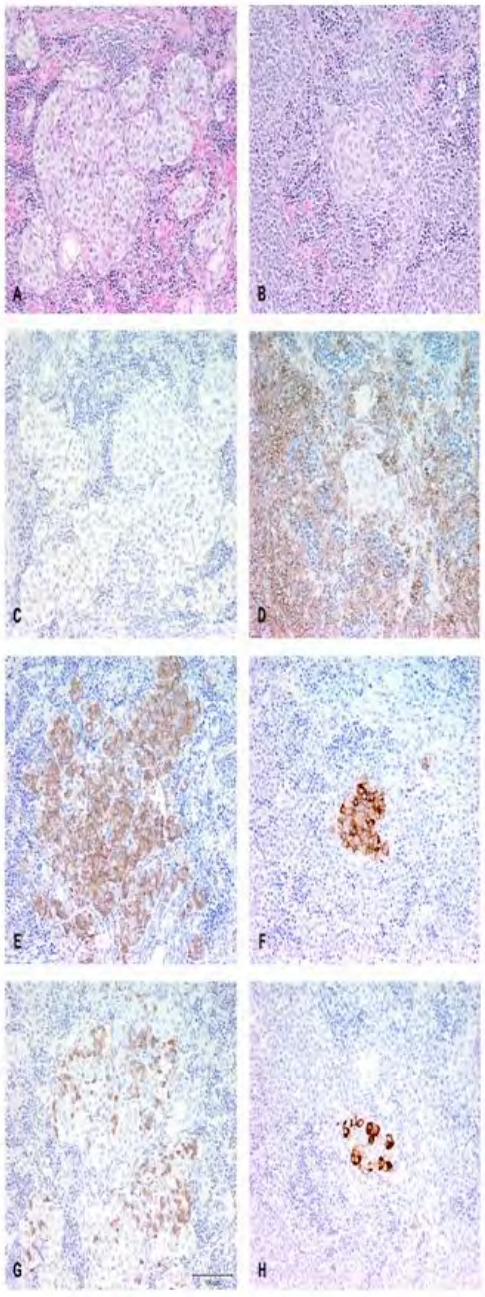


Figure 7

Figure 7. Human islet allograft histology

Human islets were transplanted into the spleen of chemically diabetic NOD-*scid IL2rγ*^{null} mice. These mice either received no further treatment (left column) or were injected intravenously on the same day with $20x10^6$ allogeneic PBMC (right column). Sections were stained with H&E (A and B), anti-human CD45 (C and D), anti-insulin (E and F) or anti-glucagon (G and H).

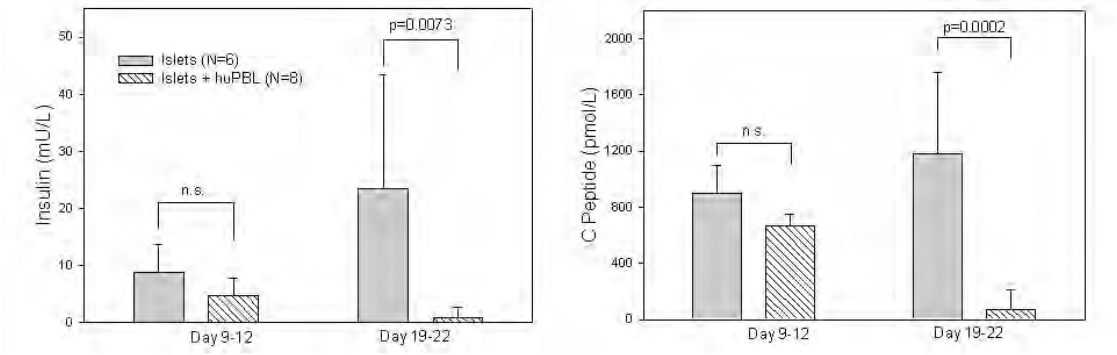


Figure 8. Serum insulin and C-peptide in human islet transplanted NOD-*scid* *IL2rγ*^{null} mice

Serum samples were collected from mice that received human islet transplants alone (solid bars; n=6) or in conjunction with 20x10^6 allogeneic PBMC (striped bars; n=8). Levels of human insulin (A) and C-peptide (B) were determined at early (day 9-12) or late (19-22) time points. Panel A: Levels of human insulin were similar at 9-12 days (9±5 *vs.* 5±3 mU/L, p=ns), but became statistically different by 19-22 days (23±20 *vs.* 1±2 mU/L, p=0.0073). Panel B: Serum C-peptide levels were similar at 9-12 days (600±376 *vs*. 493±253 pmol/L, p=ns), but became statistically different by 19-22 days (1172±572 *vs*. 73±136 pmol/L , p=0.0002).

9. Raptiva modulates PBMC engraftment in NOD-scid IL2rγ^{null} mice.

Having established that human PBMC can home to and destroy allogeneic human islets transplanted within the spleen of NOD-*scid IL2rγ^{null}* mice, investigations into mechanisms used by therapeutic agents to modulate allograft rejection are now possible. The initial question we addressed was the effect the therapeutic agent has on the ability of PBMC to engraft in NOD-*scid IL2rγ^{null}* mice. For these preliminary studies, we chose to investigate the mechanism(s) of action of Raptiva.

Raptiva, or Efalizumab, is a humanized IgG1 anti-human LFA-1 mAb. The antibody interrupts the binding of LFA-1 to its receptor, intercellular adhesion molecule-1 (ICAM-1). LFA-1 is the only integrin expressed on all leukocyte lineages (156), and as such it plays many roles in the setting of an immune response. Functions associated with LFA-1 include mediating the intercellular interactions needed for T lymphocyte-mediated cytotoxicity, lymphocyte responses, and trafficking of leukocytes into inflamed tissues **(**157-159). Its binding partner, ICAM-1, is expressed on vascular endothelial and epithelial cells (160,161). The potential for targeting LFA-1/ICAM-1 interactions as an immunotherapy for enhancing other agents for prolonging islet allograft survival has recently been reviewed (162).

Raptiva is currently approved for the clinical treatment of psoriasis and in kidney transplantation. Clinical trials are now underway to test if Raptiva can enhance islet allograft survival, prevent rheumatoid arthritis, or prevent graft-versus-host disease.

In murine models, anti-mouse LFA-1 has been shown to modulate T cell activation, migration, and islet allograft rejection. More recently, LFA-1 has been found

to function as a costimulatory molecule (162). Administration of blocking antibodies to mouse LFA-1 has been used to enhance solid organ and cell transplants, including islets (163-166). Unfortunately, Raptiva does not bind mouse LFA-1, and cross-reacts poorly with non-human primates. However, human LFA-1 and mouse ICAM-1 interact effectively, obviating issues of species-specificity (167). These two facts make the Hu-PBL-NOD-*scid IL2rγ*^{null} model an ideal system with which to investigate the mechanisms by which anti-human LFA-1 mAb mediates its actions *in vivo*.

We have begun to investigate the effect of Raptiva on human PBMC engraftment and migration. NOD-*scid IL2rγ*^{null} mice were injected intravenously with $20x10^6$ human PBMC. Group 1 received only PBMC (labeled PBL day 14 and PBL day 28 on Figure 9A and PBL on Figure 9B). Group 2 was given Raptiva (200mg) at the time of PBMC injection (day 0) and on days 1 and 7 after PBMC injection (labeled anti-LFA-1 day 0 on graph). Group 3 was given Raptiva on days 14 and 21 after PBMC injection (labeled anti-LFA-1 day 14 on graph).

The percentage of human $CD45^+$ cells in the blood of Group 1 at day 14 was 9.4±6.3% and increased to 50.9±14.3% on day 28 (Figure 9A). In contrast, mice given Raptiva beginning on the day of PBMC injection (Group 2) had few (0.3±0.3%) human $CD45^+$ cells in their blood on day 28 ($p<0.0001$ vs. Group 1). Mice permitted to engraft for 2 weeks prior to injection of Raptiva on days 14 and 21 (Group 3) had an intermediate level of human $CD45^+$ cells in their blood (24.2±12.6%, $p<0.01$ vs. Group 1 and $p<0.005$ vs. Group 2, Figure 9A). The percentage of human $CD45^+$ cells in the spleen on day 28 in Group 1 was 62.8±10.7% (Figure 9B). Mice given Raptiva beginning on the day of

PBMC injection (Group 2) had few (2.6±2.9%) human $CD45^+$ cells in the spleen on day 28 ($p<0.0001$ vs. group 1). In contrast, mice permitted to engraft for 2 weeks prior to injection of Raptiva on days 14 and 21 (Group 3) had an high level of human $CD45^+$ cells in their spleen on day 28 (57.5±14.7%) that was equivalent to that observed in mice not given Raptiva (p=NS vs. Group 1 and p=0.0002 vs. Group 2, Figure 9B).

These data provide insights into the mechanisms by which Raptiva functions *in vivo*. First, Raptiva prevents the activation and/or expansion of naïve human T cells in vivo as Group 2 had low levels of human PBMC at day 28 as compared to untreated controls (Group 1). Second, Raptiva is not a depleting antibody as initiation of treatment on day 14 (Group 3) after allowing human PBMC to engraft and expand in the mice does not deplete the already engrafted human cells. Third, Raptiva prevents the migration of human T cells from the spleen into the blood. The blood levels of human $CD45^+$ cells in Group 3 were reduced whereas the levels of human $CD45^+$ cells in the spleen of Group 3 were similar to that of untreated controls (Group 1). Finally, Raptiva does not prevent the continued expansion of activated human T cells as the levels of splenic human $CD45^+$ cells were similar in Groups 1 and 3.

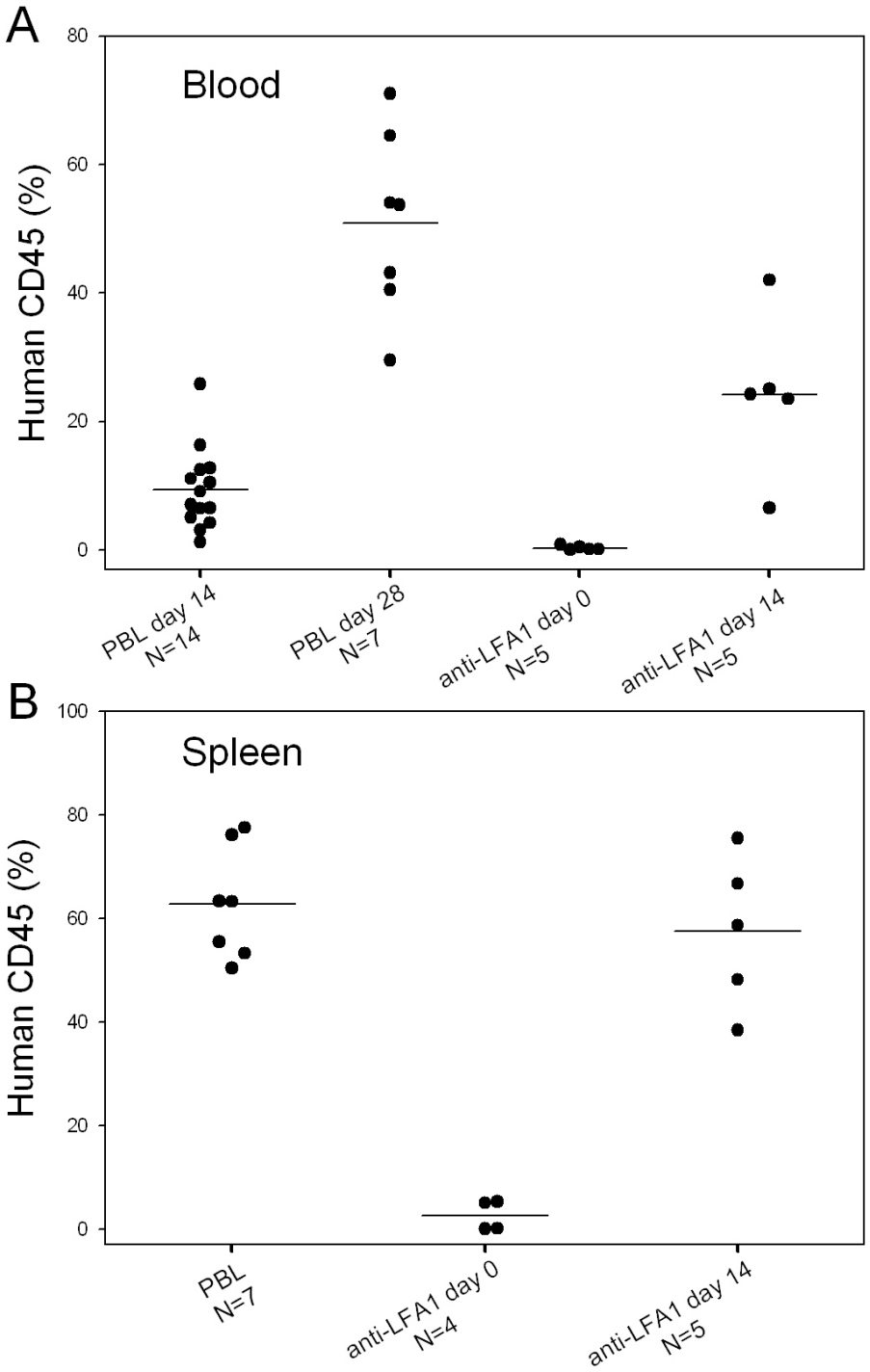

A
Blood
Human CD45 (%)
80
60
40
20
0
PBL day 14
N=14
PBL day 28
N=7
anti-LFA1 day 0
N=5
anti-LFA1 day 14
N=5
B
Spleen
Human CD45 (%)
100
80
60
40
20
0
PBL
N=7
anti-LFA1 day 0
N=4
anti-LFA1 day 14
N=5

Figure 9

Figure 9. Modulation of human PBMC engraftment and migration in Hu-PBL-SCID mice injected with anti-LFA-1.

NOD-*scid IL2rγ*^{null} mice were injected intravenously with 20x10^6 human PBMC prepared from two different donors. Panel A: PBL day 14 and PBL day 28 received only PBMC (Group 1 in text) and were analyzed on day 14 and 28, respectively. Anti-LFA-1 day 0 (Group 2 in text) was given Raptiva (200μg) at the time of PBMC injection (day 0) and on days 1 and 7 after PBMC injection and analyzed on day 28. Anti-LFA-1 day 14 (Group 3 in text) was given Raptiva on days 14 and 21 after PBMC injection and analyzed on day 28. Panel B: PBL (Group 1), anti-LFA-1 day 0 (Group 2) and anti-LFA-1 day 14 (Group 3) are the same mice as in Panel A and were analyzed on day 28.

SUMMARY

In this chapter, we demonstrate the improved engraftment of human PBMC in NOD-*scid* *IL2rγ*^{null} mice compared to the previous "gold standard" NOD-*scid* strain. We then defined the optimal route of injection, optimal cell dose and the kinetics of human cell engraftment into NOD-*scid IL2rγ*^{null} mice. The functionality of engrafted human T-cells was demonstrated by the ability of engrafted human PBMC to target allogeneic human islets transplanted into the spleen of chemically diabetic NOD-*scid IL2rγ*^{null} mice. Finally, in preparation for *in vivo* studies investigating the mechanisms by which Raptiva prevents allograft rejection, we describe the effects of Raptiva on the engraftment of human PBMC. We determined that Raptiva prevents activation or proliferation of naïve T-cells but not activated T-cells. Further, Raptiva does not deplete T-cells *in vivo*, but does impact the ability of T-cells to migrate from the spleen.

Together, the data presented in Chapter 4 describe a new model system for mechanistic studies of human allograft rejection, and how potential therapeutics may affect these mechanisms *in vivo*.

CHAPTER V: XENOGRAFT-VERSUS-HOST DISEASE IN THE HU-PBL-NOD-*SCID IL2Rγ*^{NULL} MODEL

INTRODUCTION

Graft versus host disease remains the major limitation to the success of allogeneic bone marrow transplantation (99). The development of a small animal model of the human immune system for the study of GVHD has followed a similar path as the development of models of human allorejection described in the introduction.

For example, human PBMC engraftment in mice harboring the *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency) mutation (120) only occurred at very low levels, and then only after i.p. injection of a high number of human PBMC ($50x10^6$ to $100x10^6$ cells). Most of the human cells that did engraft became anergic (168,169). Only a small percentage of these unconditioned CB17-*scid* mice engrafted with human PBMC developed a GVHD-like disease (170), making the Hu-PBL-*scid* mouse a poor model for this particular line of research.

The NOD-*scid* strain of mice have many defects in the innate immune function that together allow for higher human PBMC engraftment compared to CB17-*scid* mice (171). However, the level of engrafted human cells remained quite low (172). As the level of T cell chimerism has been directly correlated with the development of GVHD (170), it is not surprising that only a fraction of NOD-*scid* mice injected with PBMC develop symptoms consistent with clinical GVHD.

As the primary barrier to achieving higher levels of human cell engraftment in immunodeficient mice remains NK cells, several groups have reported efforts to decrease the activity of this cell population by targeted cell depletion (173,174) or genetic

modification (175,176). Recently, two approaches have been reported to genetically impair the development of NK cells in mice. First, an $H2^d$ mouse deficient in both *Rag2* and the common gamma chain of the IL-2 receptor (*IL2rγ*) had some advantages compared to NOD-*scid* mice. A higher level of human cell engraftment was reached, and engraftment was successful with 90% of human PBMC donors tested. However, extensive preconditioning of the recipient with total body irradiation and macrophage depletion using clodronate-containing liposomes was required and still did not eliminate the variability in human cell engraftment and the development of GVHD symptoms in these mice (175).

A second approach to genetically inhibit the maturation of NK cells was based on a NOD-*scid* $\beta 2m^{null}$ mouse (176). This strain of mouse was able to support human cell engraftment and the development of GVHD without the requirement for macrophage depletion. However, retro-orbital injection of $10x10^6$ purified human T-lymphocytes into lightly irradiated mice produced GVHD in only 59% of the animals whereas PBMC injection via the tail vein exhibited only transient engraftment and failed to induce GVHD (176).

Immunodeficient NOD-*scid* $lL2r\gamma^{null}$ readily engraft with human PBMC. In Chapter 5, we report a new human into mouse xenograft model of GVHD based on intravenous injection of PBMCs via the tail vein into lightly conditioned NOD-*scid* $IL2r\gamma^{null}$ mice. These mice consistently (100%) develop GVHD following injection of as few as $5x10^6$ PBMC, regardless of the PBMC donor used. As in human disease, the development of GVHD was highly dependent on host expression of MHC class I and

class II molecules. Interrupting the TNFα signaling cascade with etanercept, a therapeutic in clinical trial for human GVHD, delayed the onset and progression of disease.

RESULTS

1. Low dose irradiation accelerates GVHD in NOD-scid IL2rγ^{null} mice injected with human PBMC

NOD-*scid IL2rγ^{null}* mice engrafted with human PBMC develop a gradual weight loss and exhibit hunched posture, anemia, decreased mobility and ruffled fur (177), symptoms that are consistent with GVHD. A recently reported human into mouse xenograft model of GVHD describes the use of total body irradiation and host macrophage depletion as requisite preconditioning for consistent induction of GVHD (175). As we have observed untreated NOD-*scid IL2rγ^{null}* mice more readily engraft with high levels of human PBMC than do BALB/c-*Rag1^{null} IL2rγ^{null}* mice and eventually develop GVHD symptoms (177), we asked whether low dose irradiation alone would accelerate the onset and progression of GVHD.

NOD-*scid IL2rγ^{null}* mice given 2 Gy of total body irradiation and injected intravenously via the tail vein with $20x10^6$ human PBMC developed an accelerated form of GVHD leading to decreased survival as compared to mice that received PBMC in the absence of irradiation preconditioning. (**Figure 10A**. MST=11 *vs*. MST=34 days; $p<0.0001$). As expected, 2 Gy sublethal irradiation had no effect on the survival of otherwise untreated mice (**Figure 10A**). To determine if weight loss was a sensitive predictor of GVHD, irradiated and non-irradiated NOD-*scid IL2rγ^{null}* mice were injected with $20x10^6$ human PBMC and weighed three times weekly for approximately 6 weeks. We found that weight loss was a strong predictor of survival in mice engrafted with human PBMC (**Figure 10B**).

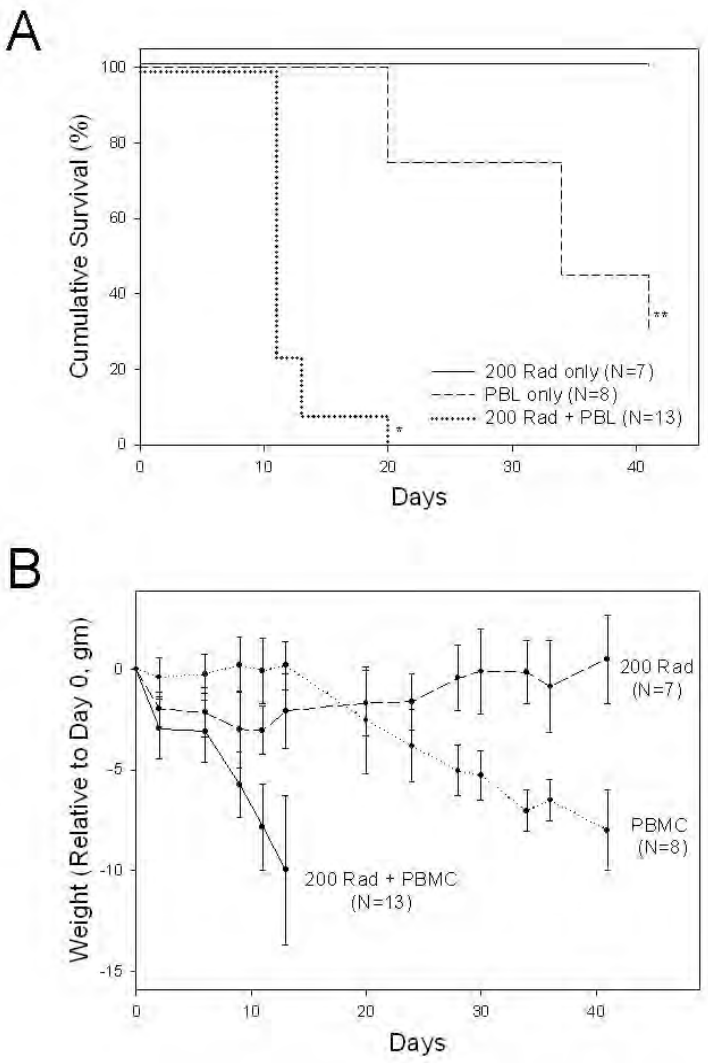

A
Cumulative Survival (%)
100
80
60
40
20
0
0
10
20
30
40
Days
200 Rad only (N=7)
PBL only (N=8)
200 Rad + PBL (N=13)
**
*
B
Weight (Relative to Day 0, gm)
0
-5
-10
-15
0
10
20
30
40
Days
200 Rad
(N=7)
PBMC
(N=8)
200 Rad + PBMC
(N=13)

Figure 10

Figure 10. Sublethal irradiation accelerates GVHD in Hu-PBMC-NOD-scid IL2rγ^{null} Mice.

NOD-*scid IL2rγ^{null}* mice received either 2 Gy irradiation only (solid line, n=7), 20x10^6 human PBMC i.v. (dashed line, MST=34 days, n=8, 3 PBMC donors), or both 2 Gy irradiation and PBMC (dotted line, MST=11, n=13, 3 PBMC donors). (A) Survival of irradiated, PBMC injected, mice was significantly shorter that mice receiving PBMC alone ($p<0.0001$). (B) Changes in total body weight relative to weight at the beginning of the experiment are shown.

2. Kinetics of GVHD development is dependent on inoculum cell dose

We next determined the effect of varying PBMC cell dose on the development of GVHD. Published models of xeno-GVHD in irradiated immunodeficient mice have injected between $15x10^6$ and $30x10^6$ human PBMC and have inconsistent induction of GVHD symptoms (175,178). However, we have observed that as few as $5x10^6$ PBMC consistently results in engraftment in non-irradiated NOD-*scid IL2rγ*^{null} mice (177). To determine the effect of cell dose on disease development, we injected $5x10^6$, $10x10^6$ or $20x10^6$ human PBMC intravenously into 2 Gy irradiated mice. There was a strong correlation between the median survival time (MST) and cell dose (Figure 11; MST=22 days, 17 days, and 12 days, respectively). However, all mice given even the low dose of $5x10^6$ from multiple donors developed GVHD.

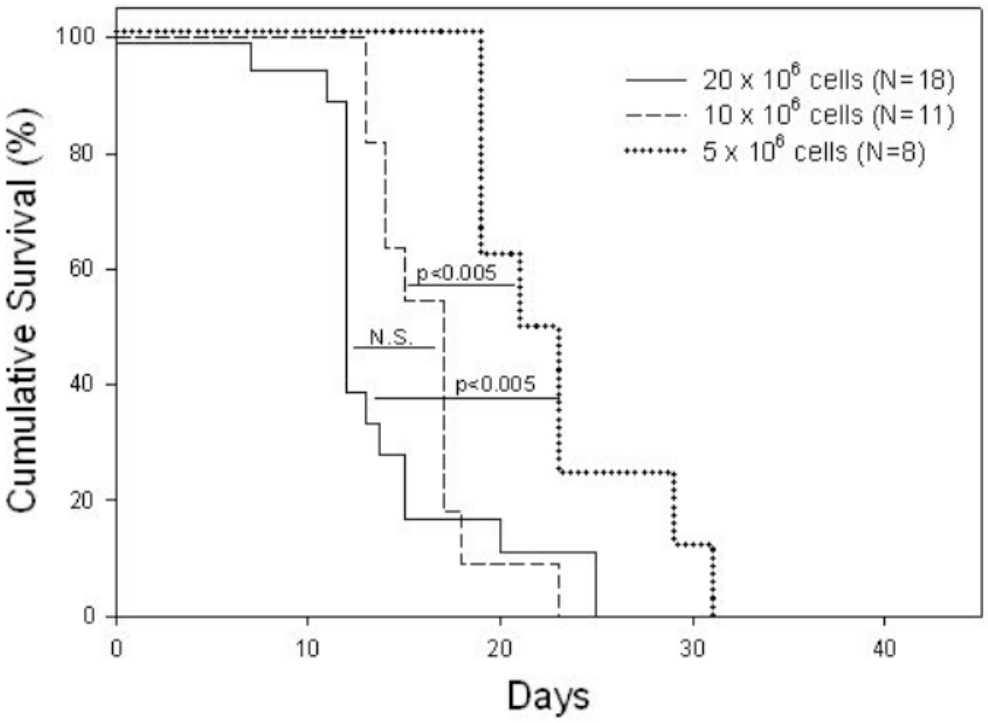


Figure 11. GVHD progression is dependent on PBMC cell dose.

Survival of NOD-*scid IL2rγ*^{null} mice that received 200 cGy TBI prior to injection of $20x10^6$ (solid line, MST=12 days, n=18, 4 PBMC donors), $10x10^6$ (dashed line, MST=17 days, n=11, 4 PBMC donors) or $5x10^6$ (dotted line, MST=22 days, N=8, 3 PBMC donors), human PBMC i.v.

3. Murine MHC stimulates human PBMC proliferation in vitro

It has been hypothesized that human cell $CD4^+$ T cell expansion in immunodeficient mice is driven by xenoreactivity to mouse MHC class II (168). However, in previous models using CB17-*scid* or NOD-*scid* mice, $CD8^+$ T cells were found to predominate in the engrafted recipient (131,132,143). Furthermore, in NOD-*scid IL2rγ*^{null} mice, both $CD4^+$ and $CD8^+$ T cells engraft at high levels (177) suggesting that reactivity to both mouse MHC class I and class II may occur.

To begin to determine the role of host MHC on human PBMC engraftment, we first determined the percentage of human T cells that proliferate *in vitro* in response to murine MHC class I and class II molecules. CFSE-labeled human PBMC were cultured alone or in the presence of splenocytes as antigen-presenting cells that were obtained from NOD-*scid*, NOD-*scid β2m*^{null} (MHC class I deficient), NOD-*scid* A_B^{null} (MHC class II deficient), or NOD-*scid β2m*^{null} A_B^{null} (MHC class I and class II deficient) mice. As expected (168) a lower proportion of human $CD4^+$ cells proliferated in response to NOD-*scid* A_B^{null} and NOD-*scid β2m*^{null} A_B^{null} (both MHC class II deficient) splenocytes as compared to NOD-*scid* splenocytes ($p<0.025$ and 0.05, respectively, Figure 12, left panel). Alternatively, a lower proportion of human $CD8^+$ cells proliferated in response to NOD-*scid β2m*^{null} and NOD-*scid β2m*^{null} A_B^{null} (both MHC class I deficient) splenocytes as compared to NOD-*scid* splenocytes (both $p<0.025$, Figure 12, right panel). These data suggest that some, but not all proliferation of human T cells in immunodeficient NOD-*scid* mice is in response to reactivity to host MHC class I and class II molecules.

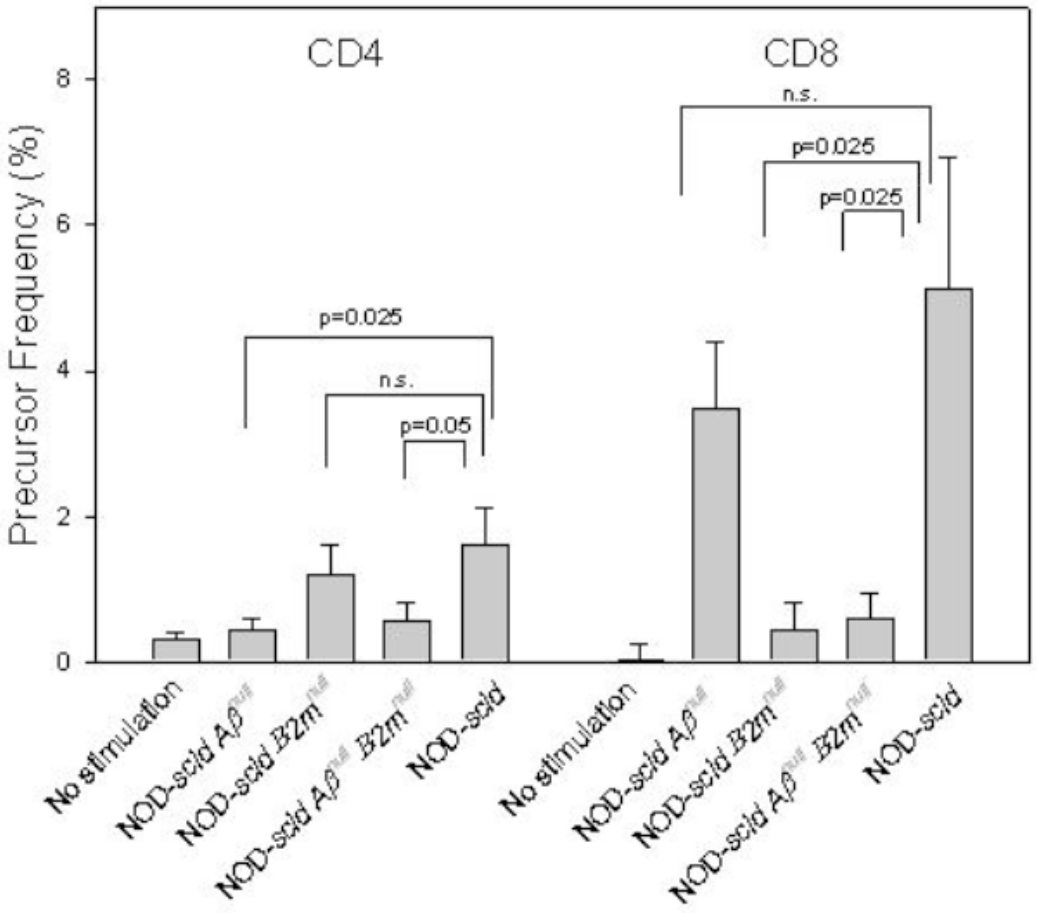


Figure 12. In vitro proliferation of human PBMC in response to murine class I or class II MHC. CSFE-labeled human PBMC were cultured alone or in the presence of APC splenocytes obtained from NOD-*scid*, NOD-*scid* $\beta 2m^{null}$, NOD-*scid* A_{β}^{null} or NOD-*scid* $\beta 2m^{null}$ A_{β}^{null} mice for 7 days. The precursor frequency index of one experiment representative of 3 independent experiments is shown (± 1 s.d.).

4. Recognition of host MHC class I drives the development of GVHD

We found that a higher proportion of human PBMC proliferate in response to *in vitro* stimulation with cells expressing murine MHC class I as compared to stimulator cells expressing murine MHC class II (Figure 12). Based on this observation and the previous report that host MHC class II drove human T cell proliferation (168), we hypothesized that mice lacking MHC Class I or class II expression would exhibit delayed development of GVHD. To test this, NOD-*scid IL2rγ*^{null}*β2m*^{null} (deficient in MHC class I) or NOD-*scid-IL2rγ*^{null}*A*$_{\beta}^{null}$ mice (deficient in MHC class II) were irradiated with 2 Gy and injected with 5x10^6 human PBMC. Surprisingly, we observed that NOD-*scid-IL2rγ*^{null} *A*$_{\beta}^{null}$ mice were highly susceptible to GVHD, although disease progression was slightly but significantly delayed as compared to that observed in NOD-*scid IL2rγ*^{null} mice expressing both MHC molecules (Figure 13, MST=22 *vs.* 21 days, respectively, p=0.02). More interestingly, NOD-*scid IL2rγ*^{null}*β2m*^{null} mice were relatively resistant to disease development (MST=44 days), which was significantly delayed relative to that observed in NOD-*scid IL2rγ*^{null} *A*$_{\beta}^{null}$ (p=0.001) and NOD-*scid IL2rγ*^{null} (p<0.0001) mice.

To determine if decreased human CD45$^+$ cell engraftment was the mechanism by which GVHD was significantly delayed in NOD-*scid IL2rγ*^{null}*β2m*^{null} mice, peripheral blood was analyzed at the time of euthanasia of animals exhibiting GVHD. Decreased human CD45$^+$ cell engraftment was observed in both NOD-*scid IL2rγ*^{null}*β2m*^{null} mice (13.9±6.9%, p=0.007) and NOD-*scid IL2rγ*^{null} *A*$_{\beta}^{null}$ mice (18.1±8.3%, p=0.006) compared to mice expressing both MHC molecules (53.6±4.4%, **Figure 14A**). Surprisingly, human CD45$^+$ cell engraftment in mice deficient in MHC class I was not

significantly different from that seen in mice deficient in MHC class II (p=N.S.). As expected, we observed that NOD-*scid IL2rγ*^{null}*β2m*^{null} null mice have significantly higher CD4/CD8 ratios in the blood (p=0.01), spleen (p=0.01) and bone marrow (p=0.0009) than the other two strains (**Figure 14B**).

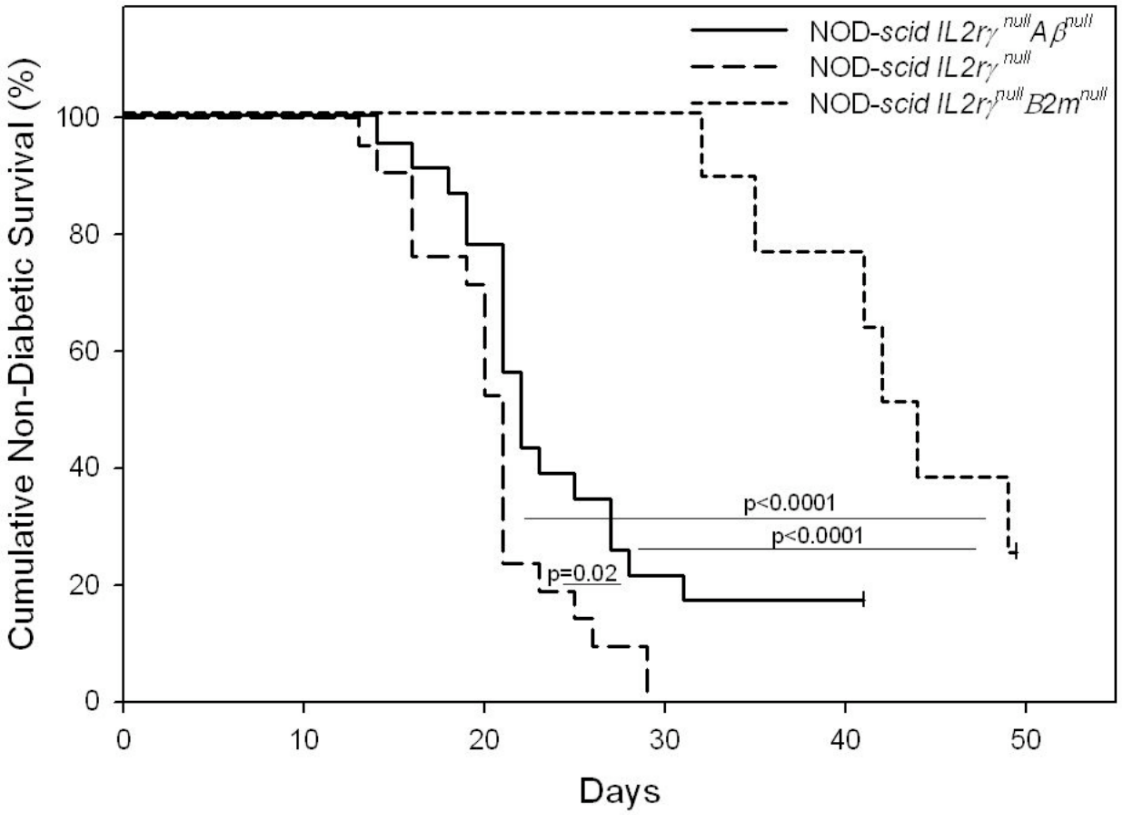
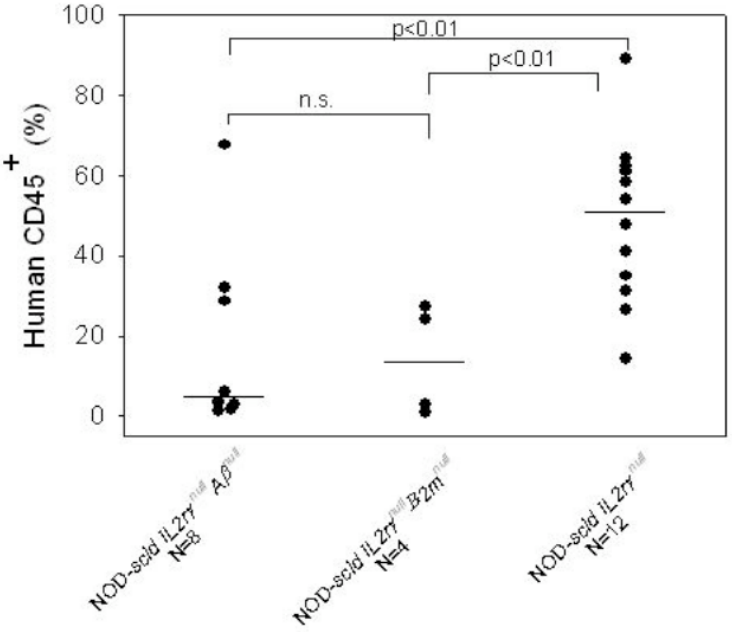


Figure 13. Mice deficient in MHC Class I expression are resistant to GVHD.

Survival of NOD-*scid IL2rγ*^{null} (dashed line, MST=21 days, n= 21, 6 PBMC donors), Nod-*scid IL2rγ*^{null}*A*$_{\beta}^{null}$ (solid line, MST=22 days, n=23, 6 PBMC donors) and NOD-*scid IL2rγ*^{null}*B2m*^{null} (dotted line, MST=46.5 days, n=10, 4 PBMC donors) following TBI and i.v. injection of 5x10^6 cells.

A


Human CD45+ (%)
100
80
60
40
20
0
p<0.01
p<0.01
n.s.
NOD-scid IL2rγnull Aβnull N=8
NOD-scid IL2rγnull B2mnull N=4
NOD-scid IL2rγnull N=12

B

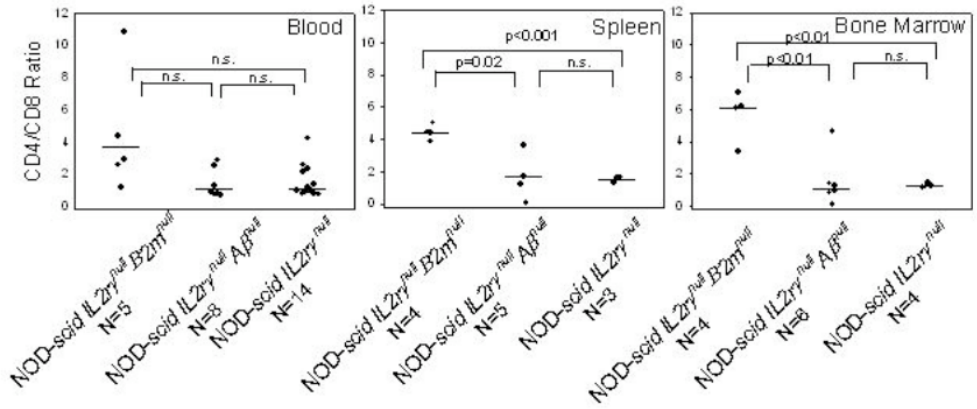

CD4/CD8 Ratio
Blood
n.s.
n.s.
n.s.
NOD-scid IL2rγnull B2mnull N=5
NOD-scid IL2rγnull Aβnull N=8
NOD-scid IL2rγnull N=14
Spleen
p<0.001
p=0.02
n.s.
NOD-scid IL2rγnull B2mnull N=4
NOD-scid IL2rγnull Aβnull N=5
NOD-scid IL2rγnull N=3
Bone Marrow
p<0.01
p<0.01
n.s.
NOD-scid IL2rγnull B2mnull N=4
NOD-scid IL2rγnull Aβnull N=6
NOD-scid IL2rγnull N=4

Figure 14

Figure 14. Decreased PBMC engraftment in MHC deficient mice

Engraftment of Nod-*scid IL2rγ*^{null} , Nod-*scid IL2rγ*^{null}*Aβ*^{null} and Nod-*scid IL2rγ*^{null}*B2m*^{null} mice that received 2 Gy and i.v. injection of $5x10^6$ cells was determined. Peripheral blood, spleen and bone marrow of moribund mice were analyzed. (A) Engraftment of human $CD45^+$ cells in the peripheral blood of moribund mice. (B) Ratio of the percent of human $CD4^+$ *vs.* human $CD8^+$ cells recovered from the peripheral blood, spleen and bone marrow of moribund mice.

5. Soluble TNFα receptor delays progression of GVHD

TNFα has been reported to have an important role in the pathogenesis of GVHD (90,179-181). In Phase I and II clinical trials, anti-TNFα antibody treatment decreased the severity of disease (182). Similarly, blocking TNFα with etanercept, (Enbrel, a soluble TNFα receptor) as part of a GVHD treatment regimen was shown to be efficacious (183). To determine if etanercept would delay or prevent GVHD, we first treated NOD-*scid* *IL2rγ*^{null} mice with two doses of etanercept injected i.p. prior to irradiation and intravenous injection of $20x10^6$ human PBMC. Pretreatment with etanercept significantly increased the survival of mice from a MST of 12 to 16 days, with one mouse surviving to day 41 (**Figure 15A**, p=0.007). To determine if a more aggressive drug regimen would prolong survival further, mice were injected with etanercept every 3 days following PBMC injection in addition to the pretreatment dosing. This dosing regimen increased survival (MST = 23 days) significantly over the two dose regimen (**Figure 15A**, p=0.01). For all groups, weight loss consistently correlated with disease progression (**Figure 15B**).

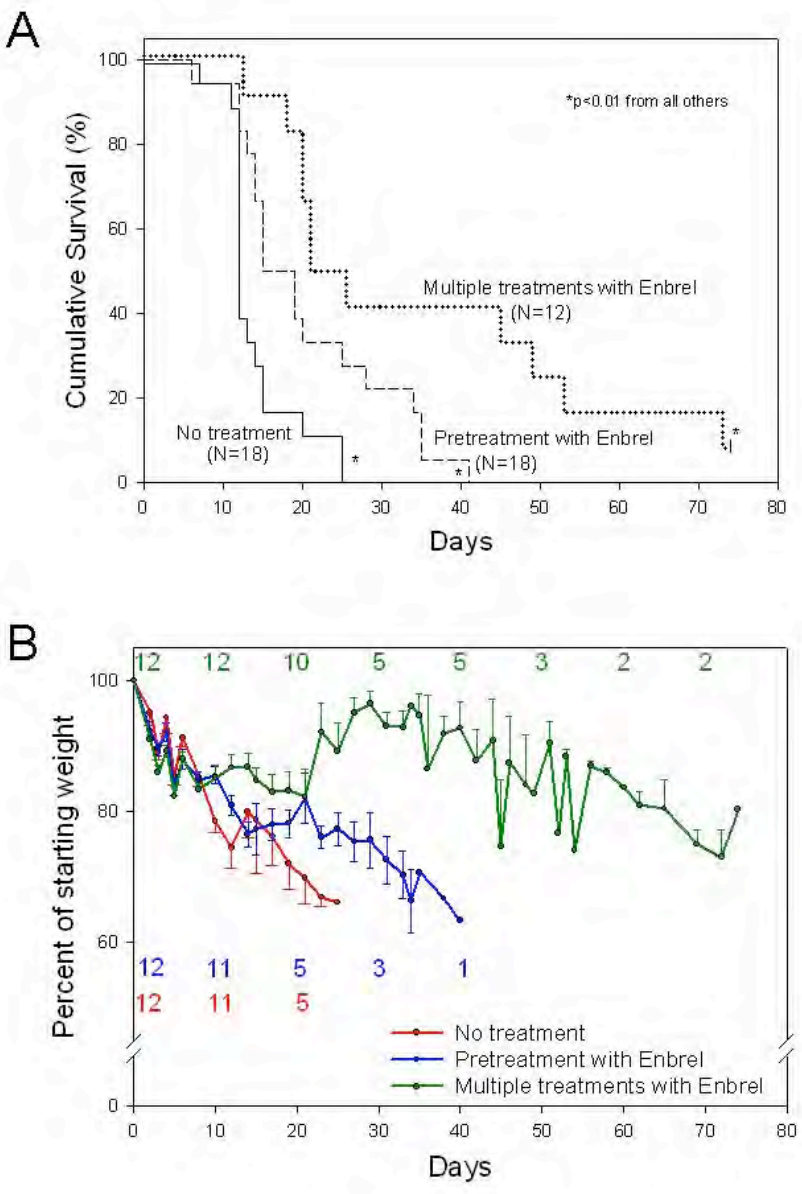

A
Cumulative Survival (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Days
*p<0.01 from all others
Multiple treatments with Enbrel
(N=12)
No treatment
(N=18)
Pretreatment with Enbrel
(N=18)
*
B
Percent of starting weight
12 12 10 5 5 3 2 2
12 11 5 3 1
12 11 5
No treatment
Pretreatment with Enbrel
Multiple treatments with Enbrel

Figure 15

Figure 15. Soluble TNFα receptor delays progression of GVHD.

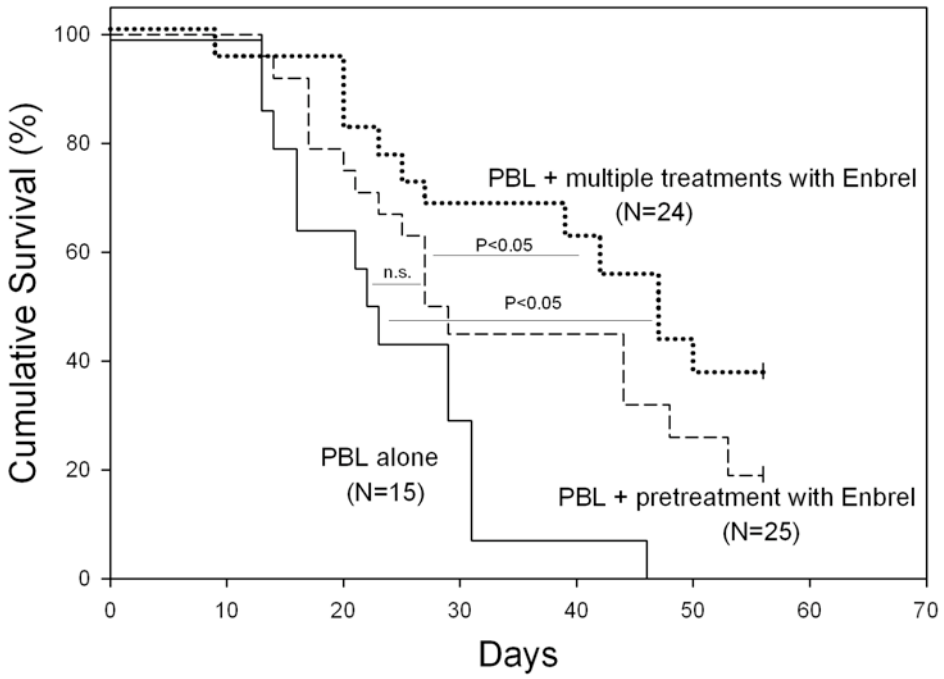
(A) Survival of NOD-*scid IL2rγ*^{null} mice that received 20x10^6 PBMC i.v. four hours after 2 Gy TBI (solid line, MST=12 days, n=18, 7 PBMC donors), with etanercept pretreatment of 100 μg/injection on days -3 and -1 prior to injection of PBMC (dashed line, MST= 16 days, n=18, 7 PBMC donors) or etanercept pretreatment plus 100 μg every three days (MST=23 days, n= 12, 4 PBMC donors). (B). The average weight change in irradiated mice that received PBMC alone (red line), PBMC plus etanercept pretreatment (blue line) or etanercept pretreatment plus 100 μg every three days (green line) is shown as the percent of starting weight. The numbers shown correspond with the surviving mice at each time point.

6. Etanercept-dependent disease prevention at lower PBMC doses

The injection of $20x10^6$ human PBMC led to a robust GVHD with no mouse surviving past day 25 (**Figure 15A**). We next determined the effect of both etanercept dosing schedules on GVHD development in mice injected with $5x10^6$ human PBMC. All mice given etanercept exhibited delays in the development of GVHD (**Figure 16**). Both the two dose etanercept pretreatment and repeat dose etanercept treatment significantly increased survival compared to mice receiving only $5x10^6$ PBMC ($p=0.03$ and $p=0.0003$ respectively). However, we observed no statistical difference in survival between the two treatment groups (**Figure 16A**, $p=0.15$). Interestingly, 3 of 25 mice that were pretreated with etanercept as well as 6 of 24 mice that received repeat doses of etanercept remained GVHD-free at the end of the observation period.

One possible mechanism by which etanercept could delay disease development would be to reduce human PBMC engraftment. To investigate this, the percentage of human cells engrafted in the mice was determined 14 days after irradiation with 2 Gy and injection of $5x10^6$ human PBMC alone or in combination with the two etanercept dosing regimens. The percentage of human $CD45^+$ cells was significantly decreased in the peripheral blood ($p<0.0001$), spleen ($p<0.0001$) and bone marrow ($p<0.0001$) in mice given either etanercept dosing regiment as compared to non-drug treated mice (**Figure 16B**).

A


Cumulative Survival (%)
Days
PBL + multiple treatments with Enbrel
(N=24)
P<0.05
n.s.
P<0.05
PBL alone
(N=15)
PBL + pretreatment with Enbrel
(N=25)

B

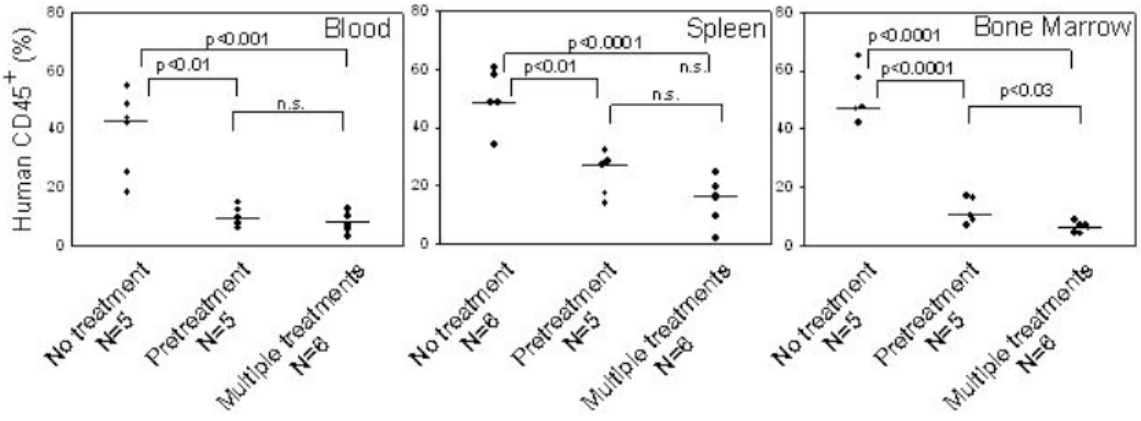

Human CD45+ (%)
Blood
p<0.001
p<0.01
n.s.
Spleen
p<0.0001
p<0.01
n.s.
n.s.
Bone Marrow
p<0.0001
p<0.0001
p<0.03
No treatment N=5
Pretreatment N=5
Multiple treatments N=6
No treatment N=6
Pretreatment N=5
Multiple treatments N=6
No treatment N=5
Pretreatment N=5
Multiple treatments N=6

Figure 16

Figure 16. Etanercept-dependent disease prevention at lower PBMC doses.

(**A**) Survival of NOD-*scid IL2rγ*^{null} mice that received 5x10^6 PBMC i.v. four hours after 200 cGy TBI (solid line, MST=22 days, n=15, 7 PBMC donors), with etanercept pretreatment of 100 μg/injection on days -3 and -1 prior to injection of PBMC (dashed line, MST=28 days, n=25, 7 PBMC donors) or etanercept pretreatment plus additional 100 μg etanercept injections every three days (dotted line, MST=47 days, n=24, 7 PBMC donors). PBMC *vs* etanercept pretreatment, p=0.03; PBMC vs multidose etanercept, p=0.0003; etanercept pretreatment vs multidose etanercept, p=ns. (**B**) Mice received 2 Gy four hours prior to 5x10^6 PBMC. 14 days later, human CD45$^+$ cell engraftment in the blood (left panel), spleen (middle panel) and bone marrow (right panel) was evaluated by flow cytometry. Etanercept treatment deceased human CD45$^+$ engraftment in all tissues tested ($p<0.0001$).

7. Infiltration of human CD45$^+$ cells in peripheral tissues

Previous reports have described infiltration of human cells into various tissues of immunodeficient mice developing GVHD-like symptoms (175). To evaluate human cell infiltration in irradiated NOD-*scid IL2rγ^{null}* mice injected with human PBMC, spleen, gut, lung, liver, skin and tongue of mice developing clinical symptoms of GVHD were studied histologically. Human CD45$^+$ cells were detected in the spleen, gut, lung and liver (data not shown). There were 3 mice that received 5x10^6 PBMC along with the multidose etanercept treatment regimen that began to lose fur 50-60 days after PBMC injection. Interestingly, in one mouse that was losing fur, we observed infiltration of human cells into the skin immediately in the area of hair loss (**Figure 17**). The infiltrate of human lymphocytes is present at the dermal epidermal junction (**Figure 17B**), and consists of both CD4$^+$ (**Figure 17D**) and CD8$^+$ (**Figure 17E**) lymphocytes in a lichenoid pattern with cells arranged in a banded configuration parallel to the epidermis.

8. Expression of human cytokines in mice with GVHD

There has been conflicting reports regarding the production of human cytokines in humanized mice. For example, in their H2^d *Rag2^{null}γc^{null}* based model, van Rijn *et al* (175) reported detectable levels of human IFNγ, GM-CSF, IL-6, IL-10, IL-13, high levels of IL-1α, IL-2, IL-15 and IL-18. The presence of IL-4, IL-8 and TNFα were not detected. In contrast, human IFNγ and IL-1β, but not IL-2, IL-4, IL-6, IL-10 and TNFα, were detected in the NOD-*scid β2m^{null}* model of GVHD reported by Nervi *et al*, (176). To determine the levels of human cytokines in the plasma of etanercept-treated or untreated NOD-*scid IL2rγ^{null}* mice we recovered plasma at various times after PBMC injection and

determined the level of human cytokines using a flow cytometry-based methodology (184).

With the exception of one animal in the PBMC alone group, we did not detect human IL-1β, IL-6, IL-10 or IL12-p70 above background levels in either etanercept treated or untreated mice at any time point tested (data not shown). IL-8 was detected at low levels in all mice at 1 hour and 4 hour timepoints, but was not detectable at the 24 hours after PBMC injection.

Plasma levels of human TNFα in mice that received PBMC alone were not statistically different from background levels from mice that did not receive PBMC (**Figure 18).** Similarly, human TNFα levels in mice treated with etanercept were not statistically different from PBMC alone mice at 1 hour (12.7+/-12.4 vs 7.2+/- 6.4 pg/ml; n=6, p=0.39) or 2 hours post-PBMC injection (22.4+/- 14.8 vs 9.5+/-14.6 pg/ml; p=0.2). Surprisingly, 24 hours after PBMC injection, plasma levels of human TNFα were significantly higher in etanercept treated mice compared to mice that received PBMC alone (33.94+/-15.10 pg/ml vs 9.433+/-16.01 pg/ml; p=0.03).

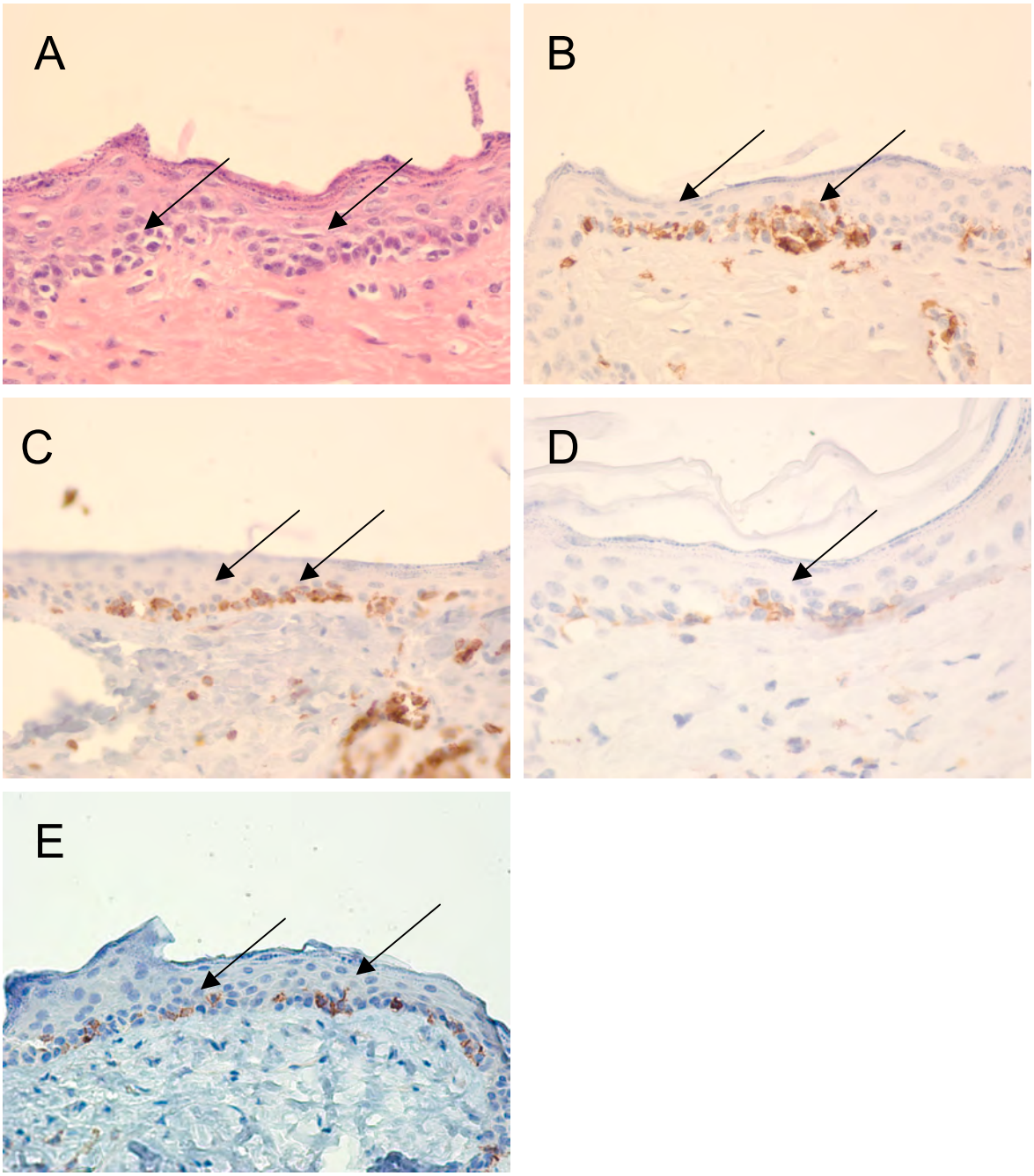


Figure 17

Figure 17. Infiltration of human lymphocytes in the skin of NOD-*scid IL2rγ^{null}* mice.

Truncal skin from NOD-*scid IL2rγ^{null}* mice 63 days after irradiation with 2 Gy and injection with $5x10^6$ human PBMC. Infiltrate of lymphocytes is present at the dermal epidermal junction. Arrows point to human cell infiltration. (A) H&E (B) human CD45 (C) human CD3 (D) human CD4 (E) human CD8. (400X)

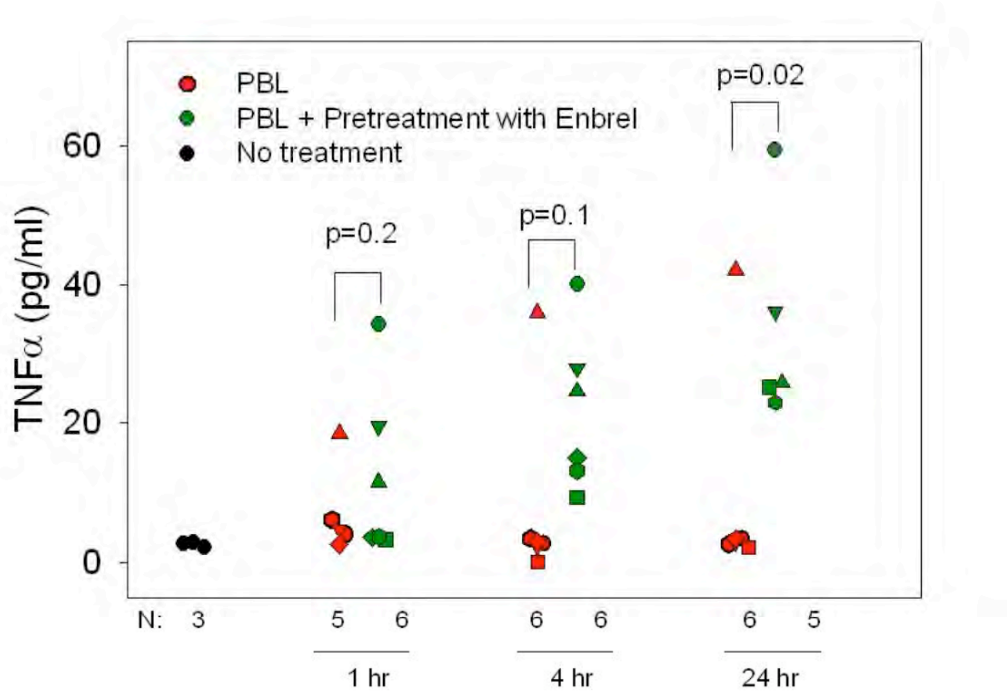


Figure 18. Plasma levels of human TNFα in etanercept-treated mice.

Levels of human TNFα in plasma samples collected from untreated or etanercept-pretreated mice 1 hour, 4 hours and 24 hours after PBMC injection. All mice received 2 Gy 4 hours prior to i.v. injection of $5x10^6$ PBMC from one of two PBMC donors. Each dot represents one mouse.

SUMMARY

In this chapter, we present evidence that low-dose total body irradiation of NOD-*scid Il2rγ*^{null} mice prior to intravenous injection with human PBMC results in a model of GVHD that develops more consistently than previously reported models. We also addressed the important role host MHC plays in the in the development of GVHD in this model. We then demonstrate that the disease that develops in NOD-*scid Il2rγ*^{null} mice mimics the clinical disease in its response to soluble TNFα receptor, etanercept, and begin to investigate the mechanism underlying this activity.

CHAPTER VI: DISCUSSION

An in vivo model of human allorejection

In Chapter IV, we report the development and characterization of the latest-generation Hu-PBL-SCID mouse model, which takes advantage of the NOD-*scid lL2rγ*^{null} strain and highlights its utility in a human islet allograft rejection system. We document the superior human PBMC engraftment in these mice and the corollary improvement in the robustness of the rejection of allogeneic human islet grafts compared to previous immunodeficient hosts (138).

Superior engraftment with human PBMC

Immunodeficient mice with targeted mutations at the IL-2 receptor common γ chain locus (*IL2rγ*^{null}) mice are gaining popularity as an immunodeficient host and have been shown to support high levels of human hematopoietic stem cell engraftment (152). Therefore, we sought to evaluate the NOD-*scid IL2rγ*^{null} mouse for its ability to support human PBMC engraftment. We first demonstrate that the NOD-*scid IL2rγ*^{null} mouse strain is a superior model for the engraftment of human PBMC as compared to the NOD-*scid* strain (127). This is due in part to the complete absence of T and B lymphocytes in the NOD-*scid IL2rγ*^{null} (149), *i.e*. no *scid* "leakiness" (132,172). More importantly, the *IL2rγ*^{null} mutation completely blocks the development of host NK cells, a cell population that has been previously shown to be a barrier to human hematopoietic cell engraftment (125,126,140).

Importantly, NOD-*scid IL2rγ*^{null} mice achieve high levels of human lymphohematopoietic cell engraftment following i.v. PBMC injection, a route of

administration that has not been routinely successful in NOD-*scid* mice (127,153,154). In fact, i.v. injection of PBMC in NOD-*scid IL2rγ*^{null} mice resulted in significantly higher levels of human cell engraftment than either the i.p. or i.s. route of injection of PBMC. In addition to the improved levels of human PBMC engraftment, there are multiple reasons that i.v. injection of human cells into host mice is preferable to other injection routes. First, i.v. injection opens up the possibility of broader experimental manipulations for delivery of PBMC that does not require the surgery used for intrasplenic injection into the circulation. Second, i.v. injection puts human PBMC directly into the circulation whereas following i.p. injection, 7-14 days are required for human PBMC to drain from the peritoneal cavity into the circulation (131,140). Third, i.v. injection permits immediate interaction between the human immune system with the allograft, a situation that more accurately mimics the environment observed in clinical human transplantation protocols. Thus, the ability to engraft high levels of human PBMC at low cell doses following i.v. injection will permit experimental manipulations not previously possible in earlier generations of Hu-PBL-SCID mouse models (127).

Investigating the kinetics of human cell engraftment revealed that the human CD45^{+} cells present in the peripheral blood of the host mice increased throughout the four week observation period. However, both the percentages and numbers of human CD45^{+} cells in the spleen reached a plateau three weeks after PBMC injection, suggesting that homeostatic regulatory mechanisms may remain intact, even in this xenograft model system. In agreement with the notion of homeostatic regulation, increasing the initial i.v.

PBMC inoculum over $20x10^6$ cells did not result in increased levels of human $CD45^+$ cells present at four weeks (data not shown).

The ratio of human $CD4^+$ to $CD8^+$ cells recovered from the spleens of engrafted mice stabilized at an approximate ratio of 1:1 by the second week. Evaluation of the numbers of $CD4^+$ and $CD8^+$ human cells indicate that this change in CD4/CD8 ratio was primarily due to a relative increase in the absolute number of both the $CD4^+$ and $CD8^+$ cells over time (data not shown). The relative ratio of CD4:CD8 T cells more closely mimics that observed in the circulation of the PBMC donors rather than the skewed ratio towards $CD8^+$ T cells previously observed in CB17-*scid* or NOD-*scid* PBMC engraftment models (131,132,143).

Two limitations of previously reported models for human PBMC engraftment in immunodeficient *IL2rγ*^{null} mice have been the requirements for injection of large numbers of human cells and/or the necessity for irradiation or other preconditioning regimen of the host mice to achieve engraftment (175,178). In studies using $H2^d$ *Rag2*^{null} *IL2rγ*^{null} mice, human PBMC engraftment required 3.5 Gy irradiation and treatment with chlodronate-containing liposomes to kill host monocytes and macrophages (and presumably human monocytes within the injected human PBMC population). Additionally, many of these mice rapidly developed a xenogeneic graft-versus-host disease and no functional analyses of the engrafted human immune system were performed in these studies. In contrast, all NOD-*scid IL2rγ*^{null} mice injected with $20x10^6$ human PBMC engrafted at high levels, with no graft-versus-host disease symptoms observed within the first 30 days, creating a window of opportunity for the study of the human immune response.

Furthermore, the successful engraftment of human hematolymphoid cells following injection of as few as $5x10^6$ PBMC without the need for host preconditioning represents a substantial improvement over earlier models where $50x10^6$ or more cells were injected, yet variability in engraftment remained. Indeed, injection of as few as $10x10^6$ PBMC into NOD-*scid IL2rγ*^{null} mice results in engraftment of 100% of the animals, emphasizing the dramatically lower PBMC dose needed for experimental protocols.

Large variations in engraftment levels obtained when using different PBMC donors, and in multiple mice injected with PBMC from the same donor has remained an obstacle to a reliable and reproducible small animal model of human immunity (185-188), even in H2^d *Rag2*^{null} *IL2rγ*^{null} mice (175). The NOD-*scid IL2rγ*^{null} mouse has now overcome this limitation, with remarkably low variability in engraftment. We also achieve comparable levels of engraftment in multiple experiments using the same donor. Furthermore, previous reports indicate that PBMC obtained from a subset of donors fail to result in engraftment at all in immunodeficient mice (185). We have not observed this phenomenon in this model, as PBMC from all donors utilized to date have resulted in engraftment. The implication of improved engraftment at lower cell doses and with minimized recipient to recipient and donor to donor variability is that this model will be a much more stable platform for experimental manipulation. For example, at cell doses of $10\text{-}20x10^6$ PBMC per recipient, a sizable cohort of mice can be engrafted from the same donor using a routine venipuncture blood draw, thus eliminating the confounding variables associated with cohorts of recipients engrafted from multiple blood donors (which were required at the higher PBMC doses used to achieve engraftment in previous

models). In addition, we observed a significant expansion of the injected human PBMC, suggesting proliferation of the injected cells.

Modeling islet allograft rejection

Having concluded that NOD-*scid IL2rγ*^{null} mice are superior hosts for human PBMC engraftment, we sought to test the functional aspects of the human immune system using a model of islet allograft rejection in humanized mice. In previous models of allorejection of human tissues in immunodeficient mice, both human islet grafts (138) and human skin grafts (189-191) are inconsistently or incompletely rejected by HLA-mismatched human PBMC. As a consequence, rejection often was determined *post-facto* by histopathological evidence, making this model difficult for use in interventional studies. This failure of complete rejection has been attributed in part to poor engraftment of human monocytes (192), presumably resulting in inefficient activation of the engrafted T cells. Inefficient T cell activation could render these cells anergic (150,168,169) and thus unable to mediate graft rejection. Consistent with this, we do observe a shift in the phenotype of engrafted T cells from expression towards CD45RO over time in mice that receive PBMC. The engrafted human PBMC, however, are not anergic and are able to completely reject HLA-mismatched human islet grafts in the spleens of recipient mice. Alternatively, the more physiological CD4:CD8 T cell ratio observed in PBMC-engrafted NOD-*scid IL2rγ*^{null} mice may also be responsible for the more robust rejection by retaining the $CD4^+$ T cells needed to provide "help" to efficiently activate alloreactive cytotoxic CD8 T cells that destroy the islet graft.

Interestingly, we also observed that our model would permit the engraftment of the human islets that have healed-in and restored normoglycemia prior to injection of human PBMC to mediate allograft rejection. This is an important advance, because the quality of human islets for research can be inconsistent and thus a confounding variable when PBMC and islets are co-engrafted as in previous models. This variation of the model now permits the function of the human islets to first be confirmed *in vivo* prior to the injection of human PBMC to study islet rejection.

A model of human GVHD

In Chapter V, we describe a robust model of GVHD based on the NOD-*scid* *IL2rγ*^{null} stock of mice. These mice consistently develop GVHD following low dose irradiation and intravenous injection of low numbers of PBMC. Using this model, we documented the relative contribution of host MHC class I and class II to human cell engraftment and disease expression using unique newly generated stocks of MHC-deficient NOD-*scid IL2rγ*^{null} mice. This new model of GVHD was then used to demonstrate that etanercept mediates its protective effect in part by decreasing the engraftment of human PBMC and blocking the TNFα signaling pathway.

We have previously shown NOD-*scid IL2rγ*^{null} mice to be superior hosts in their ability to support human PBMC engraftment relative to NOD-*scid* mice or BALB/c-*Rag1*^{null} *IL2rγ*^{null} mice (177,193). In unconditioned NOD-*scid IL2rγ*^{null} mice injected intravenously with $20x10^6$ human PBMC, we observed the gradual development of symptoms consistent with GVHD over the course of 4 to 6 weeks. To investigate whether we could optimize this model for the study of GVHD, we first determined the effects of

preconditioning with low dose irradiation. It has been reported the development of GVHD in other model systems is enhanced by administration of 3-3.5 Gy whole body irradiation (175,178,194). However, even in mice given irradiation pre-conditioning, engraftment and GVHD development was inconsistent and relatively high numbers of PBMC were required (175,178) or GVHD was observed following only very specific injection routes and cell doses (194). For example, a GVHD model based on the NOD-*scid* $\beta 2m^{null}$ mouse exhibited disease only after retro-orbital but not intravenous tail vein injection of human PBMC (194). Furthermore, a minimum inoculum of $10x10^6$ purified T cells was required for mice given 2.5 Gy of irradiation, and then only 59% of the mice developed the disease. In contrast to the previously reported models of GVHD, we observed that 100% of NOD-*scid IL2r*γ^{null} mice treated with low dose irradiation (2 Gy) and injected with $20x10^6$ human PBMC consistently developed disease between 10 and 20 days. Furthermore, we observed that 100% of irradiated mice developed GVHD following intravenous injection with as few as $5x10^6$ PBMC and that this effect was not donor dependent.

Contribution of host MHC to GVHD development

Using this optimized model, we next investigated the mechanism driving the engraftment of human PBMC and the development of GVHD in irradiated NOD-*scid IL2r*γ^{null} mice. It has previously been suggested that the development of GVHD is dependent on the relative engraftment of human T lymphocytes which is likely driven by recognition of host MHC (170). In murine allo-GVHD models, mice deficient in MHC class I or class II develop delayed $CD8^+$ or $CD4^+$ mediated GVHD, respectively (195),

suggesting that alloreactivity to both host MHC alleles is important in disease pathogenesis. In human-mouse xenoreactive models, early experiments evaluating human T cells isolated from CB17-*scid* mice demonstrated that human $CD4^+$ T cells recovered from engrafted mice had TCRs specific for murine MHC class II (168). However, the predominance of $CD8^+$ cells in previous models of human PBMC engraftment suggests a strong reactivity against host MHC class I may also be present (131,132,143). Furthermore, the high levels of engraftment of both $CD4^+$ and $CD8^+$ human cells observed in NOD-*scid IL2r*γ^{null} mice (177) suggests that reactivity to both host MHC class I and class II may occur.

To investigate the relative role of murine host MHC class I and class II in human cell proliferation, we first stimulated human PBMC *in vitro* with murine splenocytes isolated from mice genetically deficient in each of these molecules. As expected (168), a lower precursor frequency of human $CD8^+$ cells was observed following stimulation by splenocytes deficient in murine MHC class I, and a lower precursor frequency of human $CD4^+$ cells was observed following stimulation by splenocytes deficient in murine MHC class II. *In vivo*, the delay in GVHD observed in human PBMC-engrafted mice lacking MHC class I or class II was also consistent with host MHC playing a major role in triggering disease development. Comparison of human cell engraftment in these strains revealed a decrease in human $CD45^+$ cells in both MHC class I or class II deficient mice as compared to as NOD-*scid IL2r*γ^{null} mice that express both sets of molecules.

Surprisingly, however, there was no difference in $CD45^+$ cell engraftment between MHC class I and class II deficient recipients even though there was a significant

difference in the kinetics of development of GVHD. This observation suggests that human $CD45^+$ cell engraftment is not the only determinant of disease expression, and that the engraftment and activation of $CD8^+$ cells appears to have more of a role in mediating GVHD than does $CD4^+$ cells. This is also reflected in the ratio of $CD4^+$ to $CD8^+$ cells, which was highly skewed towards $CD4^+$ cells in the MHC class I deficient mice that exhibited delayed development of GVHD.

The role of cytokines in GVHD production

The role of the inflammatory cytokines, particularly TNFα in GVHD pathogenesis has been investigated both in experimental systems and in the clinic. Murine studies of GVHD have shown that TNFα has a key role in intestinal damage (196-198) and skin manifestations of GVHD (199,200). Clinical data has shown that high levels of TNFα production during conditioning regimens prior to allogeneic BMT were highly predictive of the subsequent severity of acute GVHD (201-203). Neutralizing the TNFα molecules has been shown to decrease pathology in target organs and improve survival in murine models of allogeneic GVHD (195,197,198). Similarly, clinical trials in which etanercept was used to neutralize serum TNFα in patients with acute GVHD have proven this approach efficacious when used in conjunction with methylprednisone (183,204).

Consistent with published studies (195,197,198), treatment with etanercept, a soluble TNFα decoy receptor, was able to delay progression of the GVHD in our model. Furthermore, we observed that etanercept mediates its effects in part by reducing the engraftment of human $CD45^+$ cells, a sensitive indicator of GVHD.

In agreement with previous studies (175,176), many cytokines, including human TNFα, were not detected in plasma samples of PBMC-injected mice. Interestingly, plasma levels of human TNFα were higher in etanercept treated compared to untreated mice (**Figure 18**). It is possible that the anti-TNFα antibody binds to an epitope distinct from the binding site of the TNF receptor binding site. Because etanercept is a recombinant homodimeric TNF receptor, the TNFα mAb used in the detection assay would recognize both receptor bound (i.e. etanercept bound), and free human TNFα in the plasma. The increase in plasma levels of TNFα over time in the etanercept treated mice could be due to TNFα/etanercept complexes that are "trapped" in the vasculature prior to clearance. The absence in TNFα in untreated mice could be a result of a "cytokine sink" phenomenon (**205**), in which the cytokine produced is rapidly being removed from the circulation by binding to its membrane-bound receptor and is thus not detectable in the plasma unless "trapped" by the soluble receptor.

In summary, in Chapter 5 we describe a robust model of GVHD disease based on the injection of human PBMC into NOD-*scid IL2rγ*^{null} mice. This model of GVHD represents several improvements over that of previously published models. First, this model reliably develops GVHD with cell-dose-dependent kinetics. Second, only minimal pre-conditioning of the murine host is required. Third, the role of host MHC can now be investigated and manipulated to suit the experimental question. Forth, disease progression responds to clinical therapeutics in a manner similar to that seen in patients. Finally, the ability to non-invasively monitor disease progression by charting weight loss over time

makes this model a powerful tool for evaluating future clinical therapeutic interventions and understanding underlying mechanisms in human GVHD.

CONCLUSIONS

Research goals

The long-term goals of our research endeavors are to develop protocols in the laboratory that can be used clinically to prolong the survival of allogeneic tissues, and decrease the occurrence of GVHD. The development of such protocols in the clinic poses many obstacles that must be overcome for success. First, clinical trials of new therapeutic agents are an expensive and lengthy undertaking. Second, efficacy trials of new therapeutics often start with patients in which current therapies were unsuccessful. As this population may prove more resistant to treatment, it will likely be more difficult to detect a therapeutic response to the intervention. Third and most important, trials of new protocols involve some degree of risk to the patient As such, there are ethical considerations to be addressed prior to starting any patient on an untested protocol and a careful weighing of the "risk to benefit" for the patient.

To minimize patient risk, extensive pre-clinical studies in laboratory animals are conducted to predict clinical responses. In the case of immunologic studies, many of these pre-clinical studies are carried out in murine models. Unfortunately, studies of murine immunity often do not predict outcomes in the clinic (106-108). To overcome this limitation, we have developed a murine model of the human alloimmune response based on the NOD-*scid IL2rγ*^{null} strain of immunodeficient mice.

A model of human alloimmunity

In Chapters 4 and 5, we presented data demonstrating the utility of NOD-*scid* *IL2rγ*^{null} mice engrafted with human PBMC to model the human alloimmune response. We showed that intravenous injection of low doses of human PBMC into NOD-*scid* *IL2rγ*^{null} mice results in highly reproducible, robust engraftment of human CD45^{+} cells. Further, the engrafted human cells are able to home to and destroy HLA-disparate human islets transplanted into the spleen. In the complete absence of host pre-conditioning, there is a 30 day "window" of opportunity to study human immunity in the absence of the confounding effects of graft-versus-host disease. Building on this observation, we modified our model system to develop a robust model of human GVHD by preconditioning the mice with 2 Gy total body irradiation prior to injection of human PBMC.

Our data demonstrate that PBMC-engrafted NOD-*scid IL2rγ*^{null} mice are an important model for investigating *in vivo* mechanisms of human islet allograft rejection and GVHD. More importantly, PBMC-engrafted NOD-*scid IL2rγ*^{null} mice provide a pre-clinical bridge for evaluating therapies without putting human subjects at risk.

Mechanisms of human cell engraftment

There are several mechanisms that may contribute to the engraftment of human PBMC in NOD-*scid IL2rγ*^{null} mice. Our data show that the primary mechanism of engraftment is via the xeno-specific proliferation of human T-cells in response to murine MHC. We have shown that a high percentage of human CD4^{+} cells (~2%) and CD8^{+} cells (~6%) proliferate *in vitro* in response to murine MHC class II and class I antigens,

respectively (**Figure 12**). The higher precursor frequency of human $CD8^+$ cells compared to $CD4^+$ cells in the *in vitro* mixed lymphocyte reaction assay is consistent with the finding that $CD8^+$ T cells predominate in most murine models of human cell engraftment (131,132,143). Similarly, the decreased engraftment of human PBMC seen in mice deficient at either MHC class I or class II allele supports the hypothesis that host MHC is a primary driving force of human PBMC proliferation (**Figure 14A**). Given the stronger stimulus for human cell proliferation by murine MHC class I vs MHC class II, it is surprising that mice deficient in either MHC class I or class II engraft with similar levels of human $CD45^+$ cells. This could be due in part to a requirement for human $CD4^+$ T cell help to support $CD8^+$ T cell engraftment in immunodeficient mice (150). Specifically, lower levels of $CD4^+$ engraftment in mice deficient in MHC class II would result in a related decrease in $CD8^+$ engraftment, and an overall decrease in the levels of human $CD45^+$ cells. In contrast, in the absence of murine MHC class I, there is decreased stimulus for the proliferation of human $CD8^+$ cells, resulting in lower levels of $CD8^+$ cell engraftment, and a corresponding overall decrease in human $CD45^+$ cell engraftment.

Our laboratory has previously reported that NOD-*scid* β_2m^{null} mice engraft with higher levels of $CD4^+$ cells than do NOD-*scid* mice which was thought to be due to lower NK cell activity (140). We now describe the presence of a relatively normal CD4:CD8 ratio in NOD-*scid IL2r*γ^{null} mice (**Figure 14B**). Based on the *in vitro* data presented in Figure 14 in conjunction with differences in overall engraftment of human cells in these two strains (**Figure 14A**), this normal ratio is likely due to the absence of host NK cell activity and a relatively equal expansion of each cell population in the host.

There are mechanisms other than recognition of murine MHC that are contributing to the human PBMC engraftment in NOD-*scid IL2rγ*^{null} mice. *In vitro* studies of the proliferative response elicited in human PBMC using murine cells as a source of antigen demonstrated that proliferation occurs in the absence of both murine MHC class I and class II (**Figure 12**). This could be due to additional, non-MHC antigens evoking a proliferative response from the PBMC. *In vivo*, an additional mechanism may be proliferation as a result of homeostatic expansion (206-208).

To determine the mechanism(s) of human cell proliferation *in vivo*, additional phenotyping of the engrafting and proliferating human cells would need to be performed. T cells that have undergone homeostatic expansion *in vivo* acquire a phenotype very similar to memory T cells (reviewed in 209). To differentiate between homeostasis-driven and activation-induced proliferation of human T-cells in this system, antigen-stimulated T cells have been reported to express the transferrin receptor, CD71, whereas T cells expanding by homeostatic mechanisms do not (210). In addition, antigen-driven T-cell expansion induces cellular enlargement that is not seen in T cells expanded via homeostatic mechanisms (210). Determination of the contribution of homeostatic versus antigen-driven proliferation of human T cells *in vivo* in NOD-*scid IL2rγ*^{null} mice remains an important question.

Indirect support for the hypothesis that homeostatic regulation contributes to human cell engraftment in NOD-*scid IL2rγ*^{null} mice is presented in the analysis of the kinetics of PBMC engraftment (**Figure 3).** Human CD45^{+} cells comprise an increasing proportion of total cells recovered from NOD-*scid IL2rγ*^{null} mice over the first three

weeks following PBMC injection. However, after three weeks both the percent (**Figure 3B**) and number (**Figure 3C**) of human $CD45^+$ cells recovered from the spleen reach a plateau. This suggests that homeostatic mechanisms for regulating the T-cell compartment remain functional even in this xenoreactive system.

Finally, in the instance of the islet allorejection model presented in Chapter 4, an additional mechanism for engraftment exists. In addition to the human cells that are proliferating in response to the murine MHC, there will presumably be a separate and distinct population of PBMC that are proliferating in response to the disparate human HLA expressed on the transplanted human islets. To date, we have not specifically studied the parameters of PBMC engraftment in NOD-*scid IL2rγ*^{null} mice transplanted with human tissues, although this would be an area of great interest.

None of the proposed mechanisms for human PBMC engraftment are mutually exclusive. The engraftment of PBMC in NOD-*scid IL2rγ*^{null} mice will most likely involve all three mechanisms, with the relative contributions of homeostatic proliferation and antigen specific proliferation to antigens other than murine MHC remain to be determined.

Mechanisms of allograft rejection

In Chapter 4, we demonstrate that human PBMC injected intravenously into NOD-*scid IL2rγ*^{null} mice are able to home to and destroy allogeneic human islets transplanted in the spleen (**Figures 6-8**). An overwhelming majority of cells that engraft in NOD-*scid IL2rγ*^{null} mice are $CD3^+$ T cells. Other human cell types are present only at low levels in the blood and spleen of PBMC engrafted NOD-*scid IL2rγ*^{null} mice, although

an exhaustive search of other tissues has not been performed to date. However, it is most probable that the rejection of transplanted human islets by HLA-disparate PBMC is the result of targeting by cytotoxic human $CD3^+$ cells. However, it remains to be determined whether $CD4^+$ or $CD8^+$ cells in the absence of the other cell subset are able to mediate the rejection of allogeneic human tissues in NOD-*scid IL2rγ*^{null} mice.

We recognize that the rejection of human islets in the NOD-*scid IL2rγ*^{null} mouse by allogeneic PBMC occurs in a setting of ongoing xenoreactivity. As the goal of this research is to investigate the human alloimmune response and potential targets for modulating that response, reducing the xenoreactivity is an important goal of future studies. Based on our finding that mice deficient in MHC class I are able to engraft with human cells (**Figure 14A**) while remaining resistant to the development of GVHD (**Figure 13**), it will be particularly interesting to evaluate the ability of PBMC to reject allogeneic human islets transplanted into NOD-*scid IL2rγ*^{null} β_2m^{null} mice.

Mechanisms of GVHD

Because the development of xeno-GVHD is closely linked to the level of human cell engraftment (170), the mechanisms underlying the development of GVHD in NOD-*scid IL2rγ*^{null} mice largely mirror the mechanisms of human cell engraftment. For instance, consistent with the importance of murine MHC in human PBMC engraftment (**Figure 14A**), mice deficient in either MHC class I or class II have a delayed development of GVHD as compared to NOD-*scid IL2rγ*^{null} mice expressing both MHC class I and class II (**Figure 13**).

The effectiveness of etanercept in preventing GVHD in this model is consistent with laboratory (195,197,198) and clinical data (184,204) describing an important role for TNFα in the pathogenesis of GVHD. Although the effects of etanercept were clear (**Figures 15, 16**) the mechanism(s) underlying this effect are less clear. One possible mechanism is a decrease in human cell engraftment in etanercept-treated mice (**Figure 16B**). Another possible mechanism is the reduction of bioavailable human TNFα. Interestingly, despite the efficacy of etanercept at ameliorating GVHD in this model, human TNFα was not detected in the plasma of PBMC-engrafted mice at any time-point tested. In contrast, there were increasing levels of human TNFα detected in the plasma of etanercept-treated mice during the 24-hour observation period (**Figure 18**).

Structurally, etanercept is a fusion protein of the dimeric extracellular portion of the p75 TNF receptor fused with human IgG1. It is likely that the produced TNFα will preferentially form an etanercept/TNFα complex rather than be available for binding to cell surface receptors, which would remove TNFα from the circulation. The TNFα bound to etanercept would not be bioavailable to the donor PBMC or host cells, leading to decreased pathology in etanercept-treated mice. Furthermore, the fusion protein consists of the TNFα receptor and IgG1. The immunoglobulin portion of this protein is designed to enhance the half-life of the fusion protein in the circulation and effectively permit the detection of the produced TNFα. In PBMC recipients that have not been treated with etanercept, the absence of detectable levels of TNFα in mice is likely due to a "cytokine sink" effect, in which available cytokine is rapidly bound to cell surface receptors and utilized by cell populations, and is thus has only a very short half-life in the plasma.

Alternatively, it could be that the primary affect of etanercept is due to neutralization of murine TNFα. Although our flow based assay was specific for human TNFα, a systematic evaluation of murine cytokines and specifically murine TNFα in this model system is an important remaining question.

Remaining limitations

While the PBMC-engrafted NOD-*scid IL2rγ*^{null} mouse model presents many advances over its predecessors, there are still limitations to overcome. First, although the human PBMC inoculum contains many different populations of leukocyte, the predominant cell population to engraft efficiently in NOD-*scid IL2rγ*^{null} mice is T cells. We have hypothesized that the inability of other cell populations, such as B cells, to engraft is a function of inefficient cross-reactivity of murine cytokines, growth factors and/or survival factors with the human receptors. Efforts are currently underway to identify specific factors required for improving engraftment of cell types other than T cells.

Limitations remain in the use of this model for the study of human alloimmunity as well. For example, utilizing human islets as a target of allogeneic PBMC simplifies monitoring graft function, as blood glucose levels can easily be monitored. However, human islets are not readily available. Those samples that do become available for research use have already been passed over for clinical applications, and are of varying quality. To circumvent this issue, we are currently investigating the use of islets isolated from mice that transgenically express human HLA molecules as targets for human alloimmunity. This approach will allow a more detailed investigation into the allo-

specificity of the rejection response. Experimental questions that need to be addressed include whether human PBMC will reject HLA-mismatched (allogeneic) islets while not targeting HLA matched (syngeneic) islets

Finally, the cost of increased engraftment of human cells in this model is the ensuing increase in GVHD. The development of lethal GVHD in PBMC engrafted NOD-*scid IL2rγ*^{null} mice beginning about 5 weeks after PBMC injection into unconditioned mice limits the timeframe that allograft rejection studies can be done. However, adding total body irradiation to the protocol has provided the most robust and reliable model of human into mouse xeno-GVHD described to date.

Despite these remaining limitations, the utility of PBMC-engrafted NOD-*scid IL2rγ*^{null} mice to model the human alloimmune response remains promising. This model system represents a cutting-edge approach to the age-old problem of testing the efficacy of clinical therapeutics without putting patients at risk.

CHAPTER VII: REFERENCES

1. Majno G. *The Healing Hand. Man and Wound in the Ancient World.* Cambridge, MA: Cambridge, MA..
2. Tilney, N. *Transplant: From Myth to Reality*. New Haven, Yale University Press.
3. Doyle, A. M., R. I. Lechler, and L. A. Turka. 2004. Organ transplantation: halfway through the first century. *J.Am.Soc.Nephrol.* 15:2965-2971.
4. Carrel, A. 1983. Landmark article, Nov 14, 1908: Results of the transplantation of blood vessels, organs and limbs. By Alexis Carrel. *JAMA* 250:944-953.
5. Hugh E.Stephenson, Jr. M. a. R. S. K. M. *America's First Nobel Prize in Medicine or Physiology:The Story of Guthrie and Carrel*. *Boston: Midwestern Vascular Surgery Society*.
6. MEDAWAR, P. B. 1991. The Nobel Lectures in Immunology. The Nobel Prize for Physiology or Medicine, 1960. Immunological tolerance. *Scand.J.Immunol.* 33:337-344.
7. MEDAWAR, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. *J.Anat.* 78:176-199.
8. Gibson, T. and P. B. MEDAWAR. 1943. The fate of skin homografts in man. *J.Anat.* 77:299-310.
9. Billingham, R. E., L. Brent, P. B. MEDAWAR, and E. M. SPARROW. 1954. Quantitative studies on tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. *Proc.R.Soc.Lond B Biol.Sci.* 143:43-58.
10. Billingham, R. E., P. L. KROHN, and P. B. MEDAWAR. 1951. Effect of cortisone on survival of skin homografts in rabbits. *Br.Med.J.* 1:1157-1163.
11. Starzl, T. E. 2000. History of clinical transplantation. *World J.Surg.* 24:759-782.
12. Owen, R. D. 1945. IMMUNOGENETIC CONSEQUENCES OF VASCULAR ANASTOMOSES BETWEEN BOVINE TWINS. *Science* 102:400-401.
13. Billingham, R. E., L. Brent, and P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature* 172:603-606.
14. Snell, G. D. 1992. The Nobel Lectures in Immunology. Lecture for the Nobel Prize for Physiology or Medicine, 1980: Studies in histocompatibility. *Scand.J.Immunol.* 36:513-526.
15. LITTLE, C. C. 1914. A POSSIBLE MENDELIAN EXPLANATION FOR A TYPE OF INHERITANCE APPARENTLY NON-MENDELIAN IN NATURE. *Science* 40:904-906.
16. Snell, G. D. and G. F. HIGGINS. 1951. Alleles at the histocompatibility-2 locus in the mouse as determined by tumor transplantation. *Genetics* 36:306-310.
17. Doherty, P. C. and R. M. Zinkernagel. 1975. H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J.Exp.Med.* 141:502-507.

18. Wilson, D. B. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of response. *J.Exp.Med.* 126:625-654.
19. Wilson, D. B., W. K. Silvers, and P. C. Nowell. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationship of the proliferative response to the immunologic status of the donors. *J.Exp.Med.* 126:655-665.
20. Wilson, D. B. and P. C. Blyth JL NOWELL. 1968. Quantitative studies on the mixed lymphocyte interaction in rats. 3. Kinetics of the response. *J.Exp.Med.* 128:1157-1181.
21. Heeger, P. S. 2003. T-cell allorecognition and transplant rejection: a summary and update. *Am.J.Transplant.* 3:525-533.
22. Selin, L. K., M. Cornberg, M. A. Brehm, S. K. Kim, C. Calcagno, D. Ghersi, R. Puzone, F. Celada, and R. M. Welsh. 2004. CD8 memory T cells: cross-reactivity and heterologous immunity. *Semin.Immunol.* 16:335-347.
23. Lindahl, K. F. and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J.Exp.Med.* 145:508-522.
24. Suchin, E. J., P. B. Langmuir, E. Palmer, M. H. Sayegh, A. D. Wells, and L. A. Turka. 2001. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J.Immunol.* 166:973-981.
25. Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman, and R. Ahmed. 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J.Exp.Med.* 195:657-664.
26. Whitmire, J. K., N. Benning, and J. L. Whitton. 2006. Precursor frequency, nonlinear proliferation, and functional maturation of virus-specific CD4+ T cells. *J.Immunol.* 176:3028-3036.
27. Starzl, T. E., T. L. Marchioro, J. H. HOLMES, G. HERMANN, R. S. BRITTAIN, O. H. STONINGTON, D. W. TALMAGE, and W. R. WADDELL. 1964. RENAL HOMOGRAFTS IN PATIENTS WITH MAJOR DONOR-RECIPIENT BLOOD GROUP INCOMPATIBILITIES. *Surgery* 55:195-200.
28. Galili, U. 1993. Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. *Immunol.Today* 14:480-482.
29. Game, D. S. and R. I. Lechler. 2002. Pathways of allorecognition: implications for transplantation tolerance. *Transpl.Immunol.* 10:101-108.
30. Benichou, G. 1999. Direct and indirect antigen recognition: the pathways to allograft immune rejection. *Front Biosci.* 4:D476-D480.
31. Caballero, A., N. Fernandez, R. Lavado, M. J. Bravo, J. M. Miranda, and A. Alonso. 2006. Tolerogenic response: allorecognition pathways. *Transpl.Immunol.* 17:3-6.
32. Womer, K. L., M. H. Sayegh, and H. Auchincloss, Jr. 2001. Involvement of the direct and indirect pathways of allorecognition in tolerance induction. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 356:639-647.

33. Lindahl, K. F. and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. I. Estimates of the absolute frequency of killer cells generated in vitro. *J.Exp.Med.* 145:500-507.
34. Hornick, P. I., P. D. Mason, M. H. Yacoub, M. L. Rose, R. Batchelor, and R. I. Lechler. 1998. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. *Circulation* 97:1257-1263.
35. Hornick, P. I., P. D. Mason, R. J. Baker, M. Hernandez-Fuentes, L. Frasca, G. Lombardi, K. Taylor, L. Weng, M. L. Rose, M. H. Yacoub, R. Batchelor, and R. I. Lechler. 2000. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation* 101:2405-2410.
36. HARRISON, J. H., J. P. Merrill, and J. E. Murray. 1956. Renal homotransplantation in identical twins. *Surg.Forum* 6:432-436.
37. MERRILL, J. P., J. E. MURRAY, J. H. HARRISON, and W. R. GUILD. 1956. Successful homotransplantation of the human kidney between identical twins. *J.Am.Med.Assoc.* 160:277-282.
38. MORGAN, J. A. 1951. The influence for cortisone on the survival of homografts of skin in the rabbit. *Surgery* 30:506-515.
39. CANNON, J. A. and W. P. LONGMIRE, Jr. 1952. Studies of successful skin homografts in the chicken; description of a method of grafting and its application as a technic of investigation. *Ann.Surg.* 135:60-68.
40. DEMPSTER, W. J., B. LENNOX, and J. W. BOAG. 1950. Prolongation of survival of skin homotransplants in the rabbit by irradiation of the host. *Br.J.Exp.Pathol.* 31:670-679.
41. MAIN, J. M. and R. T. PREHN. 1955. Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J.Natl.Cancer Inst.* 15:1023-1029.
42. Murray, J. E., J. P. Merrill, G. J. DAMMIN, J. B. DEALY, Jr., C. W. WALTER, M. S. BROOKE, and R. E. WILSON. 1960. Study on transplantation immunity after total body irradiation: clinical and experimental investigation. *Surgery* 48:272-284.
43. HAMBURGER, J., J. VAYSSE, J. CROSNIER, J. AUVERT, C. M. LALANNE, and J. HOPPER, Jr. 1962. Renal homotransplantation in man after radiation of the recipient. Experience with six patients since 1959. *Am.J.Med.* 32:854-871.
44. KUSS, R., M. LEGRAIN, G. MATHE, R. NEDEY, and M. CAMEY. 1962. Homologous human kidney transplantation. Experience with six patients. *Postgrad.Med.J.* 38:528-531.
45. HITCHINGS, G. H. and G. B. ELION. 1954. The chemistry and biochemistry of purine analogs. *Ann.N.Y.Acad.Sci.* 60:195-199.
46. Duncan, M. D. and D. S. Wilkes. 2005. Transplant-related immunosuppression: a review of immunosuppression and pulmonary infections. *Proc.Am.Thorac.Soc.* 2:449-455.

47. SCHWARTZ, R. and W. Dameshek. 1959. Drug-induced immunological tolerance. *Nature* 183:1682-1683.
48. MEEKER, W. R., Jr., R. M. Condie, R. A. Good, and R. L. VARCO. 1960. Alteration of the homograft response by antimetabolites. *Ann.N.Y.Acad.Sci.* 87:203-213.
49. SCHWARTZ, R. and W. Dameshek. 1960. The effects of 6-mercaptopurine on homograft reactions. *J.Clin.Invest* 39:952-958.
50. PIERCE, J. C. and R. L. VARCO. 1962. Induction of tolerance to a canine renal homotransplant with 6-mercaptopurine. *Lancet* 1:781-782.
51. ZUKOSKI, C. F. and J. M. CALLAWAY. 1963. Adult tolerance induced by 6-methyl mercaptopurine to a canine renal homograft. *Nature* 198:706-707.
52. Murray, J. E., A. G. Sheil, R. MOSELEY, P. KNIGHT, J. D. MCGAVIC, and G. J. DAMMIN. 1964. ANALYSIS OF MECHANISM OF IMMUNOSUPPRESSIVE DRUGS IN RENAL HOMOTRANSPLANTATION. *Ann.Surg.* 160:449-473.
53. HOPEWELL, J., R. Y. Calne, and I. BESWICK. 1964. THREE CLINICAL CASES OF RENAL TRANSPLANTATION. *Br.Med.J.* 1:411-413.
54. Starzl, T. E., T. L. Marchioro, and W. R. WADDELL. 1963. THE REVERSAL OF REJECTION IN HUMAN RENAL HOMOGRAFTS WITH SUBSEQUENT DEVELOPMENT OF HOMOGRAFT TOLERANCE. *Surg.Gynecol.Obstet.* 117:385-395.
55. Calne, R. Y. 1977. Renal transplantation. *Lancet* 2:1283-1284.
56. Calne, R. Y., D. J. White, B. D. Pentlow, K. Rolles, T. Syrakos, T. Ohtawa, D. P. Smith, P. McMaster, D. B. Evans, B. M. Herbertson, and S. Thiru. 1979. Cyclosporin A: preliminary observations in dogs with pancreatic duodenal allografts and patients with cadaveric renal transplants. *Transplant.Proc.* 11:860-864.
57. Green, C. J. and A. C. Allison. 1978. Extensive prolongation of rabbit kidney allograft survival after short-term cyclosporin-A treatment. *Lancet* 1:1182-1183.
58. Calne, R. Y., D. J. White, S. Thiru, D. B. Evans, P. McMaster, D. C. Dunn, G. N. Craddock, B. D. Pentlow, and K. Rolles. 1978. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* 2:1323-1327.
59. Calne, R. Y., K. Rolles, D. J. White, S. Thiru, D. B. Evans, P. McMaster, D. C. Dunn, G. N. Craddock, R. G. Henderson, S. Aziz, and P. Lewis. 1979. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. *Lancet* 2:1033-1036.
60. Gruessner, R. W., G. W. Burke, R. Stratta, H. Sollinger, E. Benedetti, C. Marsh, P. Stock, J. P. Boudreaux, M. Martin, M. B. Drangstveit, D. E. Sutherland, and A. Gruessner. 1996. A multicenter analysis of the first experience with FK506 for induction and rescue therapy after pancreas transplantation. *Transplantation* 61:261-273.
61. Gruessner, R. W., D. E. Sutherland, M. B. Drangstveit, C. Troppmann, and A. C. Gruessner. 1996. Use of FK 506 in pancreas transplantation. *Transpl.Int.* 9 Suppl 1:S251-S257.

62. Gruessner, R. W. 1997. Tacrolimus in pancreas transplantation: a multicenter analysis. Tacrolimus Pancreas Transplant Study Group. *Clin.Transplant.* 11:299-312.
63. Laskow, D. A., J. F. Neylan, III, R. S. Shapiro, J. D. Pirsch, P. J. Vergne-Marini, and S. J. Tomlanovich. 1998. The role of tacrolimus in adult kidney transplantation: a review. *Clin.Transplant.* 12:489-503.
64. Paraskevas, S., J. E. Coad, A. Gruessner, R. Kandaswamy, A. Humar, D. E. Sutherland, and R. W. Gruessner. 2005. Posttransplant lymphoproliferative disorder in pancreas transplantation: a single-center experience. *Transplantation* 80:613-622.
65. Starzl, T. E., S. Todo, J. Fung, A. J. Demetris, R. Venkataramman, and A. Jain. 1989. FK 506 for liver, kidney, and pancreas transplantation. *Lancet* 2:1000-1004.
66. Fung, J. J., S. Todo, A. Jain, J. McCauley, M. Alessiani, C. Scotti, and T. E. Starzl. 1990. Conversion from cyclosporine to FK 506 in liver allograft recipients with cyclosporine-related complications. *Transplant.Proc.* 22:6-12.
67. Armitage, J. M., R. L. Kormos, B. P. Griffith, R. L. Hardesty, F. J. Fricker, R. S. Stuart, G. C. Marrone, S. Todo, J. Fung, and T. E. Starzl. 1991. A clinical trial of FK 506 as primary and rescue immunosuppression in cardiac transplantation. *Transplant.Proc.* 23:1149-1152.
68. Starzl, T. E., A. Donner, M. Eliasziw, L. Stitt, P. Meier, J. J. Fung, J. P. McMichael, and S. Todo. 1995. Randomised trialomania? The multicentre liver transplant trials of tacrolimus. *Lancet* 346:1346-1350.
69. Kuo, C. J., J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree. 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* 358:70-73.
70. Lee, Y. R., I. H. Yang, Y. H. Lee, S. A. Im, S. Song, H. Li, K. Han, K. Kim, S. K. Eo, and C. K. Lee. 2005. Cyclosporin A and tacrolimus, but not rapamycin, inhibit MHC-restricted antigen presentation pathways in dendritic cells. *Blood* 105:3951-3955.
71. McGregor, D. D. and J. L. Gowans. 1964. SURVIVAL OF HOMOGRAFTS OF SKIN IN RATS DEPLETED OF LYMPHOCYTES BY CHRONIC DRAINAGE FROM THE THORACIC DUCT. *Lancet* 1:629-632.
72. Starzl, T. E., R. Weil, III, L. J. Koep, Y. Iwaki, P. I. Terasaki, and G. P. Schroter. 1979. Thoracic duct drainage before and after cadaveric kidney transplantation. *Surg.Gynecol.Obstet.* 149:815-821.
73. Starzl, T. E., G. B. Klintmalm, S. Iwatsuki, and R. Weil, III. 1981. Late follow-up after thoracic duct drainage in cadaveric renal transplantation. *Surg.Gynecol.Obstet.* 153:377-382.
74. Woodruff, M. F. and N. A. ANDERSON. 1963. EFFECT OF LYMPHOCYTE DEPLETION BY THORACIC DUCT FISTULA AND ADMINISTRATION OF ANTILYMPHOCYTIC SERUM ON THE SURVIVAL OF SKIN HOMOGRAFTS IN RATS. *Nature* 200:702.
75. Iwasaki, Y., K. A. Porter, J. R. Amend, T. L. Marchioro, V. Zuhlke, and T. E. Starzl. 1967. The preparation and testing of horse antidog and antihuman

antilymphoid plasma or serum and its protein fractions. *Surg.Gynecol.Obstet.* 124:1-24.

76. Starzl, T. E., T. L. Marchioro, K. A. Porter, Y. Iwasaki, and G. J. Cerilli. 1967. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. *Surg.Gynecol.Obstet.* 124:301-308.
77. Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495-497.
78. Hale, G., M. J. Dyer, M. R. Clark, J. M. Phillips, R. Marcus, L. Riechmann, G. Winter, and H. Waldmann. 1988. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* 2:1394-1399.
79. Vincenti, F., R. Kirkman, S. Light, G. Bumgardner, M. Pescovitz, P. Halloran, J. Neylan, A. Wilkinson, H. Ekberg, R. Gaston, L. Backman, and J. Burdick. 1998. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N.Engl.J.Med.* 338:161-165.
80. Nashan, B., R. Moore, P. Amlot, A. G. Schmidt, K. Abeywickrama, and J. P. Soulillou. 1997. Randomised trial of basiliximab versus placebo for control of acute cellular rejection in renal allograft recipients. CHIB 201 International Study Group. *Lancet* 350:1193-1198.
81. Nakakura, E. K., R. A. Shorthouse, B. Zheng, S. M. McCabe, P. M. Jardieu, and R. E. Morris. 1996. Long-term survival of solid organ allografts by brief anti-lymphocyte function-associated antigen-1 monoclonal antibody monotherapy. *Transplantation* 62:547-552.
82. Ferry, C. and G. Socie. 2003. Busulfan-cyclophosphamide versus total body irradiation-cyclophosphamide as preparative regimen before allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia: what have we learned? *Exp.Hematol.* 31:1182-1186.
83. Soulillou, J. P. and M. Giral. 2001. Controlling the incidence of infection and malignancy by modifying immunosuppression. *Transplantation* 72:S89-S93.
84. Laederach-Hofmann, K. and B. Bunzel. 2000. Noncompliance in organ transplant recipients: a literature review. *Gen.Hosp.Psychiatry* 22:412-424.
85. Bhatia, M. and M. C. Walters. 2008. Hematopoietic cell transplantation for thalassemia and sickle cell disease: past, present and future. *Bone Marrow Transplant* 41:109-117.
86. Prasad, V. K. and J. Kurtzberg. 2008. Emerging trends in transplantation of inherited metabolic diseases. *Bone Marrow Transplant* 41:99-108.
87. Burt, R. K., L. Verda, Y. Oyama, L. Statkute, and S. Slavin. 2004. Non-myeloablative stem cell transplantation for autoimmune diseases. *Springer Semin.Immunopathol.* 26:57-69.
88. Welniak, L. A., B. R. Blazar, and W. J. Murphy. 2007. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu.Rev Immunol.* 25:139-170.

89. Petersen, S. L. 2007. Alloreactivity as therapeutic principle in the treatment of hematologic malignancies. Studies of clinical and immunologic aspects of allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning. *Dan.Med.Bull.* 54:112-139.
90. Sun, Y., I. Tawara, T. Toubai, and P. Reddy. 2007. Pathophysiology of acute graft-versus-host disease: recent advances. *Transl.Res.* 150:197-214.
91. MATHE, G., J. L. AMIEL, L. SCHWARZENBERG, A. CATTAN, and M. SCHNEIDER. 1963. HAEMATOPOIETIC CHIMERA IN MAN AFTER ALLOGENIC (HOMOLOGOUS) BONE-MARROW TRANSPLANTATION. (CONTROL OF THE SECONDARY SYNDROME. SPECIFIC TOLERANCE DUE TO THE CHIMERISM). *Br.Med.J.* 2:1633-1635.
92. Billingham, R. E. 1966. The biology of graft-versus-host reactions. *Harvey Lect.* 62:21-78.
93. Starzl, T. E., K. A. Porter, G. Andres, C. G. Halgrimson, R. Hurwitz, G. Giles, P. I. Terasaki, I. Penn, G. T. Schroter, J. Lilly, S. J. Starkie, and C. W. Putnam. 1970. Long-term survival after renal transplantation in humans: (with special reference to histocompatibility matching, thymectomy, homograft glomerulonephritis, heterologous ALG , AND RECIPIENT MALIGNANCY). *Ann.Surg.* 172:437-472.
94. Anasetti, C., D. Amos, P. G. Beatty, F. R. Appelbaum, W. Bensinger, C. D. Buckner, R. Clift, K. Doney, P. J. Martin, E. Mickelson, and . 1989. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N.Engl.J.Med.* 320:197-204.
95. Petersdorf, E. W., G. M. Longton, C. Anasetti, E. M. Mickelson, S. K. McKinney, A. G. Smith, P. J. Martin, and J. A. Hansen. 1997. Association of HLA-C disparity with graft failure after marrow transplantation from unrelated donors. *Blood* 89:1818-1823.
96. Flomenberg, N., L. A. Baxter-Lowe, D. Confer, M. Fernandez-Vina, A. Filipovich, M. Horowitz, C. Hurley, C. Kollman, C. Anasetti, H. Noreen, A. Begovich, W. Hildebrand, E. Petersdorf, B. Schmeckpeper, M. Setterholm, E. Trachtenberg, T. Williams, E. Yunis, and D. Weisdorf. 2004. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood* 104:1923-1930.
97. Goulmy, E., R. Schipper, J. Pool, E. Blokland, J. H. Falkenburg, J. Vossen, A. Gratwohl, G. B. Vogelsang, H. C. van Houwelingen, and J. J. van Rood. 1996. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N.Engl.J.Med.* 334:281-285.
98. Shlomchik, W. D. 2007. Graft-versus-host disease. *Nat.Rev.Immunol.* 7:340-352.
99. Ferrara, J. L. and J. E. Levine. 2006. Graft-versus-host disease in the 21st century: new perspectives on an old problem. *Semin Hematol.* 43:1-2.
100. Lee, S. J. 2005. New approaches for preventing and treating chronic graft-versus-host disease. *Blood* 105:4200-4206.

101. Bresson, D. and M. von Herrath. 2007. Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? *Autoimmun.Rev.* 6:315-322.
102. von Herrath, M. G. and G. T. Nepom. 2005. Lost in translation: barriers to implementing clinical immunotherapeutics for autoimmunity. *J.Exp.Med.* 202:1159-1162.
103. Perel, P., I. Roberts, E. Sena, P. Wheble, C. Briscoe, P. Sandercock, M. Macleod, L. E. Mignini, P. Jayaram, and K. S. Khan. 2007. Comparison of treatment effects between animal experiments and clinical trials: systematic review. *BMJ* 334:197.
104. Talmadge, J. E., R. K. Singh, I. J. Fidler, and A. Raz. 2007. Murine models to evaluate novel and conventional therapeutic strategies for cancer. *Am.J.Pathol.* 170:793-804.
105. Monk, N. J., R. E. Hargreaves, E. Simpson, J. P. Dyson, and S. Jurcevic. 2006. Transplant tolerance: models, concepts and facts. *J.Mol.Med.* 84:295-304.
106. Rossini, A. A. 2004. From beast to bedside: a commentary. *Diabetologia* 47:1647-1649.
107. Roep, B. O. 2007. Are insights gained from NOD mice sufficient to guide clinical translation? Another inconvenient truth. *Ann.N.Y.Acad.Sci.* 1103:1-10.
108. Greiner, D. L., A. A. Rossini, and J. P. Mordes. 2001. Translating data from animal models into methods for preventing human autoimmune diabetes mellitus: *caveat emptor* and *primum non nocere*. *Clin Immunol* 100:134-143.
109. Bosma, G. C., R. P. Custer, and M. J. Bosma. 1983. A severe combined immunodeficiency mutation in the mouse. *Nature* 301:527-530.
110. Araki, R., A. Fujimori, K. Hamatani, K. Mita, T. Saito, M. Mori, Fukumura, R, M. Morimyo, M. Muto, M. Itoh, K. Tatsumi, and M. Abe. 1997. Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice. *Proc.Nat'l.Acad.Sci.* 94:2438-2443.
111. Kirchgessner, C. U., C. K. Patil, J. W. Evans, C. A. Cuomo, L. M. Fried, T. Carter, and J. M. Brown. 1995. DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science* 267:1178-1183.
112. Blunt, T., D. Gell, M. Fox, G. E. Taccioli, A. R. Lehmann, S. P. Jackson, Jeggo, and PA. 1996. Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the *scid* mouse. *Proc.Nat'l.Acad.Sci.USA* 93:10285-10290.
113. Fried, L. M., C. Koumenis, S. R. Peterson, S. L. Green, P. van Zijl, J. Allalunis-Turner, D. J. Chen, R. Fishel, A. J. Giaccia, J. M. Brown, and C. U. Kirchgessner. 1996. The DNA damage response in DNA-dependent protein kinase- deficient SCID mouse cells: replication protein A hyperphosphorylation and p53 induction. *Proc.Nat'l.Acad.Sci.USA* 93:13825-13830.
114. Jeggo, P. A., S. P. Jackson, and G. E. Taccioli. 1996. Identification of the catalytic subunit of DNA dependent protein kinase as the product of the mouse *scid* gene. *Curr.Top.Microbiol.Immunol.* 217:79-89.
115. Miller, R. D., J. Hogg, J. H. Ozaki, D. Gell, S. P. Jackson, and R. Riblet. 1995. Gene for the catalytic subunit of mouse DNA-dependent protein kinase maps to the *scid* locus. *Proc.Nat'l.Acad.Sci.USA* 92:10792-10795.

116. Taylor, P. C. *The use of SCID mice in the investigation of human autoimmune diseases*. R.G. Landes Company, Austin, TX, pp. 1-127.
117. Lieber, M. R., J. E. Hesse, S. Lewis, G. C. Bosma, N. Rosenberg, K. Mizuuchi, and M. Gellert. 1988. The defect in murine severe combined immune deficiency: joining of signal sequences but not coding segments in V(D)J recombination. *Cell* 55:7-16.
118. Malynn, B. A., T. K. Blackwell, G. M. Fulop, A. J. Furley, G. A. Rathbun, P. Ferrier, R. A. Phillips, G. D. Yancopoulos, and F. W. Alt. 1988. The *scid* defect affects the final step of the immunoglobulin VDJ recombinase mechanism. *Cell* 54:453-460.
119. Bosma, M. J. and A. M. Carroll. 1991. The SCID mouse mutant: definition, characterization, and potential uses. *Ann.Rev.Immunol.* 9:323-350.
120. Mosier, D. E., R. J. Gulizia, S. M. Baird, and D. B. Wilson. 1988. Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* 335:256-259.
121. McCune, J. M., R. Namikawa, H. Kaneshima, L. D. Shultz, M. Lieberman, and I. L. Weissman. 1988. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* 241:1632-1639.
122. Lapidot, T., F. Pflumio, M. Doedens, B. Murdoch, D. E. Williams, and J. E. Dick. 1992. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* 255:1137-1141.
123. Bosma, G. C., M. Fried, R. P. Custer, A. Carroll, D. M. Gibson, and M. J. Bosma. 1988. Evidence of functional lymphocytes in some (leaky) *scid* mice. *J.Exp.Med.* 167:1016-1033.
124. Fulop, G. M. and R. A. Phillips. 1990. The *scid* mutation in mice causes a general defect in DNA repair. *Nature* 347:479-482.
125. Shultz, L. D., P. A. Schweitzer, S. W. Christianson, B. Gott, I. B. Schweitzer, S. McKenna, L. Mobraaten, T. V. Rajan, D. L. Greiner, and E. H. Leiter. 1995. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-*scid* mice. *J.Immunol.* 154:180-191.
126. Christianson, S. W., D. L. Greiner, I. B. Schweitzer, B. Gott, G. L. Beamer, P. A. Schweitzer, R. M. Hesselton, and L. D. Shultz. 1996. Role of natural killer cells on engraftment of human lymphoid cells and on metastasis of human T-lymphoblastoid leukemia cells in C57BL/6J-*scid* mice and in C57BL/6J-*scid bg* mice. *Cell.Immunol.* 171:186-199.
127. Greiner, D. L., R. A. Hesselton, and L. D. Shultz. 1998. SCID mouse models of human stem cell engraftment. *Stem Cells* 16:166-177.
128. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, and S. Tonegawa. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.
129. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, M. Datta, F. Young, A. M. Stall, and et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855-867.
130. Martin, A., M. Valentine, P. Unger, S. W. Yeung, L. D. Shultz, and T. F. Davies. 1994. Engraftment of human lymphocytes and thyroid tissues into *scid* and *rag2*-

deficient mice: absent progression of lymphocyte infiltration. *J.Clin.Endo.Metabol.* 79:716-723.

131. Hesselton, R. M., D. L. Greiner, J. P. Mordes, T. V. Rajan, J. L. Sullivan, and L. D. Shultz. 1995. High levels of human peripheral blood mononuclear cell engraftment and enhanced susceptibility to human immunodeficiency virus type 1 infection in NOD/LtSz-*scid/scid* mice. *J.Inf.Dis.* 172:974-982.
132. Greiner, D. L., L. D. Shultz, J. Yates, M. C. Appel, G. Perdrizet, R. M. Hesselton, I. Schweitzer, W. G. Beamer, K. L. Shultz, S. C. Pelsue, J. H. Leif, and T. V. Rajan. 1995. Improved engraftment of human spleen cells in NOD/LtSz-*scid/scid* mice as compared with C.B-17-*scid/scid* mice. *Am.J.Path.* 146:888-902.
133. Kataoka, S., J. Satoh, H. Fujiya, T. Toyota, R. Suzuki, K. Itoh, and K. Kumagai. 1983. Immunologic aspects of the nonobese diabetic (NOD) mouse. *Diabetes* 32:247-253.
134. Serreze, D. V., J. W. Gaedeke, and E. H. Leiter. 1993. Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: Defective regulation of cytokine receptors and protein kinase C. *Proc.Nat'l.Acad.Sci.USA* 90:9625-9629.
135. Serreze, D. V., H. R. Gaskins, and E. H. Leiter. 1993. Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. *J.Immunol.* 150:2534-2543.
136. Baxter, A. G. and A. Cooke. 1993. Complement lytic activity has no role in the pathogenesis of autoimmune diabetes in NOD mice. *Diabetes* 42:1574-1578.
137. Serreze, D. V. and E. H. Leiter. 1988. Defective activation of T suppressor cell function in nonobese diabetic mice. Potential relation to cytokine deficiencies. *J.Immunol.* 140:3801-3807.
138. Banuelos, S. J., L. D. Shultz, D. L. Greiner, L. M. Burzenski, B. Gott, B. L. Lyons, A. A. Rossini, and M. C. Appel. 2004. Rejection of human islets and human HLA-A2.1 transgenic mouse islets by alloreactive human lymphocytes in immunodeficient NOD-scid and NOD-Rag1(null)Prf1(null) mice. *Clin.Immunol.* 112:273-283.
139. Prochazka, M., H. R. Gaskins, L. D. Shultz, and E. H. Leiter. 1992. The nonobese diabetic *scid* mouse: model for spontaneous thymomagenesis associated with immunodeficiency. *Proc.Nat'l.Acad.Sci.USA* 89:3290-3294.
140. Christianson, S. W., D. L. Greiner, R. M. Hesselton, J. H. Leif, E. J. Wagar, I. B. Schweitzer, T. V. Rajan, B. Gott, D. C. Roopenian, and L. D. Shultz. 1997. Enhanced human $CD4^+$ T cell engraftment in β2-microglobulin-deficient NOD-*scid* mice. *J.Immunol.* 158:3578-3586.
141. Rothenberg, B. E. and J. R. Voland. 1996. β_2 knockout mice develop parenchymal iron overload: A putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc.Nat'l.Acad.Sci.USA* 93:1529-1534.
142. de Sousa, M., R. Reimao, R. Lacerda, P. Hugo, S. H. E. Kaufmann, and G. Porto. 1994. Iron overload in β_2-microglobulin-deficient mice. *Immunol.Lett.* 39:105-111.

143. Shultz, L. D., S. Banuelos, B. Lyons, R. Samuels, L. Burzenski, B. Gott, P. Lang, J. Leif, M. Appel, A. Rossini, and D. L. Greiner. 2003. NOD/LtSz-Rag1nullPfpnull mice: a new model system with increased levels of human peripheral leukocyte and hematopoietic stem-cell engraftment. *Transplantation* 76:1036-1042.
144. Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu.Rev.Immunol.* 14:207-232.
145. Russell, J. H. and T. J. Ley. 2002. Lymphocyte-mediated cytotoxicity. *Annu.Rev.Immunol.* 20:323-370.
146. Uribe L, W. KI. 1998. X-linked SCID and other defects of cytokine pathways. *Semin Hematol.* 4:299-309.
147. Sugamura, K., H. Asao, M. Kondo, N. Tanaka, N. Ishii, K. Ohbo, M. Nakamura, and T. Takeshita. 1996. The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu.Rev.Immunol.* 14:179-205.
148. Asao, H., C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, and K. Sugamura. 2001. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J.Immunol.* 167:1-5.
149. Shultz, L. D., B. L. Lyons, L. M. Burzenski, B. Gott, X. Chen, S. Chaleff, M. Kotb, S. D. Gillies, M. King, J. Mangada, D. L. Greiner, and R. Handgretinger. 2005. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J.Immunol.* 174:6477-6489.
150. Wagar, E. J., M. A. Cromwell, L. D. Shultz, B. A. Woda, J. L. Sullivan, R. M. Hesselton, and D. L. Greiner. 2000. Regulation of human cell engraftment and development of EBV-related lymphoproliferative disorders in Hu-PBL-scid mice. *J.Immunol.* 165:518-527.
151. Whalen, B. J., L. C. Love, J. P. Mordes, A. A. Rossini, and D. L. Greiner. 1999. Intravital dye-labeled diabetogenic rat T cells retain dye, home to the pancreas, and induce diabetes. *Transplant.Proc* 31:1611-1614.
152. Shultz, L. D., F. Ishikawa, and D. L. Greiner. 2007. Humanized mice in translational biomedical research. *Nat.Rev.Immunol.* 7:118-130.
153. Amadori, A., A. Veronesi, V. Coppola, S. Indraccolo, M. Mion, and L. Chieco-Bianchi. 1996. The hu-PBL-SCID mouse in human lymphocyte function and lymphomagenesis studies: achievements and caveats. *Semin.Immunol.* 8:249-254.
154. Gregori, S., P. Mangia, R. Bacchetta, E. Tresoldi, F. Kolbinger, C. Traversari, J. M. Carballido, J. E. De Vries, U. Korthauer, and M. G. Roncarolo. 2005. An anti-CD45RO/RB monoclonal antibody modulates T cell responses via induction of apoptosis and generation of regulatory T cells. *J.Exp.Med.* 201:1293-1305.
155. Turgeon, N. A., S. J. Banuelos, L. D. Shultz, B. L. Lyons, N. Iwakoshi, D. L. Greiner, J. P. Mordes, A. A. Rossini, and M. C. Appel. 2003. Alloimmune injury and rejection of human skin grafts on human peripheral blood lymphocyte-

reconstituted non-obese diabetic severe combined immunodeficient beta2-microglobulin-null mice. *Exp.Biol Med.(Maywood.)* 228:1096-1104.

156. Nueda, A., M. Lopez-Cabrera, A. Vara, and A. L. Corbi. 1993. Characterization of the CD11a (alpha L, LFA-1 alpha) integrin gene promoter. *J.Biol.Chem.* 268:19305-19311.
157. Kishimoto, T. K., R. S. Larson, A. L. Corbi, M. L. Dustin, D. E. Staunton, and T. A. Springer. 1989. The leukocyte integrins. *Adv.Immunol.* 46:149-182.
158. Larson, R. S. and T. A. Springer. 1990. Structure and function of leukocyte integrins. *Immunol.Rev.* 114:181-217.
159. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301-314.
160. Xingyuan, M., Z. Wenyun, and W. Tianwen. 2006. Leukocyte function-associated antigen-1: structure, function and application prospects. *Protein Pept.Lett.* 13:397-400.
161. Dunehoo, A. L., M. Anderson, S. Majumdar, N. Kobayashi, C. Berkland, and T. J. Siahaan. 2006. Cell adhesion molecules for targeted drug delivery. *J.Pharm.Sci.* 95:1856-1872.
162. Nicolls, M. R. and R. G. Gill. 2006. LFA-1 (CD11a) as a therapeutic target. *Am.J.Transplant.* 6:27-36.
163. Rayat, G. R. and R. G. Gill. 2005. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* 54:443-451.
164. Berney, T., A. Pileggi, R. D. Molano, R. Poggioli, E. Zahr, C. Ricordi, and L. Inverardi. 2003. The effect of simultaneous CD154 and LFA-1 blockade on the survival of allogeneic islet grafts in nonobese diabetic mice. *Transplantation* 76:1669-1674.
165. Nicolls, M. R., M. Coulombe, J. Beilke, H. C. Gelhaus, and R. G. Gill. 2002. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839.
166. Nicolls, M. R., M. Coulombe, H. Yang, A. Bolwerk, and R. G. Gill. 2000. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J.Immunol.* 164:3627-3634.
167. Johnston, S. C., M. L. Dustin, M. L. Hibbs, and T. A. Springer. 1990. On the species specificity of the interaction of LFA-1 with intercellular adhesion molecules. *J.Immunol.* 145:1181-1187.
168. Tary-Lehmann, M., P. V. Lehmann, D. Schols, M. G. Roncarolo, and A. Saxon. 1994. Anti-SCID mouse reactivity shapes the human CD4+ T cell repertoire in hu-PBL-SCID chimeras. *J.Exp.Med.* 180:1817-1827.
169. Tary-Lehmann, M. and A. Saxon. 1992. Human mature T cells that are anergic in vivo prevail in SCID mice reconstituted with human peripheral blood. *J.Exp.Med.* 175:503-516.

170. Hoffmann-Fezer, G., C. Gall, U. Zengerle, B. Kranz, and S. Thierfelder. 1993. Immunohistology and immunocytology of human T-cell chimerism and graft-versus-host disease in SCID mice. *Blood* 81:3440-3448.
171. Hesselton, R. M., Cromwell, M., Shultz, L. D., and Sullivan, J. L. EBV-associated lymphoproliferative disease in human PBMC-reconstituted NOD/LtSz-*scid/scid* mice. Cold Spring Harbor Conference on Epstein-Barr Virus and Associated Diseases 1, 130. 1994.

Ref Type: Abstract

172. Greiner, D. L. and L. D. Shultz. 1998. Use of NOD/LtSz-*scid/scid* mice in biomedical research. In *NOD Mice and Related Strains: Research Applications in Diabetes, AIDS, Cancer and Other Diseases*. E. H. Leiter and M. A. Atkinson, eds. R.G. Landes Co., Austin, TX, pp. 173-204.
173. Shpitz, B., C. A. Chambers, A. B. Singhal, N. Hozumi, B. J. Fernandes, C. M. Roifman, L. M. Weiner, J. C. Roder, and S. Gallinger. 1994. High level functional engraftment of severe combined immunodeficient mice with human peripheral blood lymphocytes following pretreatment with radiation and anti-asialo GM1. *J.Immunol.Methods* 169:1-15.
174. Cao, T. and G. Leroux-Roels. 2000. Antigen-specific T cell responses in human peripheral blood leucocyte (hu-PBL)-mouse chimera conditioned with radiation and an antibody directed against the mouse IL-2 receptor beta-chain. *Clin.Exp.Immunol.* 122:117-123.
175. van Rijn, R. S., E. R. Simonetti, A. Hagenbeek, M. C. Hogenes, R. A. de Weger, M. R. Canninga-van Dijk, K. Weijer, H. Spits, G. Storm, L. van Bloois, G. Rijkers, A. C. Martens, and S. B. Ebeling. 2003. A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood* 102:2522-2531.
176. Nervi, B., M. P. Rettig, J. K. Ritchey, H. L. Wang, G. Bauer, J. Walker, M. L. Bonyhadi, R. J. Berenson, J. L. Prior, D. Piwnica-Worms, J. A. Nolta, and J. F. DiPersio. 2007. Factors affecting human T cell engraftment, trafficking, and associated xenogeneic graft-vs-host disease in NOD/SCID beta2mnull mice. *Exp.Hematol.* 35:1823-1838.
177. King, M., T. Pearson, L. D. Shultz, J. Leif, R. Bottino, M. Trucco, M. A. Atkinson, C. Wasserfall, K. C. Herold, R. T. Woodland, M. R. Schmidt, B. A. Woda, M. J. Thompson, A. A. Rossini, and D. L. Greiner. 2008. A new Hu-PBL model for the study of human islet alloreactivity based on NOD-scid mice bearing a targeted mutation in the IL-2 receptor gamma chain gene. *Clin.Immunol.* 126:303-314.
178. van Rijn, R. S., E. R. Simonetti, A. Hagenbeek, M. Bonyhadi, G. Storm, A. C. Martens, and S. B. Ebeling. 2007. Quantitative assessment of human T lymphocytes in RAG2(-/-)gammac(-/-) mice: the impact of ex vivo manipulation on in vivo functionality. *Exp.Hematol.* 35:117-127.

179. Hill, G. R., T. Teshima, A. Gerbitz, L. Pan, K. R. Cooke, Y. S. Brinson, J. M. Crawford, and J. L. Ferrara. 1999. Differential roles of IL-1 and TNF-alpha on graft-versus-host disease and graft versus leukemia. *J.Clin.Invest* 104:459-467.
180. Hill, G. R., J. M. Crawford, K. R. Cooke, Y. S. Brinson, L. Pan, and J. L. Ferrara. 1997. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* 90:3204-3213.
181. Xun, C. Q., J. S. Thompson, C. D. Jennings, S. A. Brown, and M. B. Widmer. 1994. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood* 83:2360-2367.
182. Herve, P., M. Flesch, P. Tiberghien, J. Wijdenes, E. Racadot, P. Bordigoni, E. Plouvier, J. L. Stephan, H. Bourdeau, E. Holler, and . 1992. Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acute graft-versus-host disease. *Blood* 79:3362-3368.
183. Uberti, J. P., L. Ayash, V. Ratanatharathorn, S. Silver, C. Reynolds, M. Becker, P. Reddy, K. R. Cooke, G. Yanik, J. Whitfield, D. Jones, R. Hutchinson, T. Braun, J. L. Ferrara, and J. E. Levine. 2005. Pilot trial on the use of etanercept and methylprednisolone as primary treatment for acute graft-versus-host disease. *Biol.Blood Marrow Transplant.* 11:680-687.
184. Foster, B., C. Prussin, F. Liu, J. K. Whitmire, and J. L. Whitton. 2007. Detection of intracellular cytokines by flow cytometry. *Curr.Protoc.Immunol.* Chapter 6:Unit.
185. Hesselton, R. M., R. A. Koup, M. A. Cromwell, B. S. Graham, M. Johns, and J. L. Sullivan. 1993. Human peripheral blood xenografts in the SCID mouse: characterization of immunologic reconstitution. *J.Infect.Dis.* 168:630-640.
186. Murphy, W. J., M. Bennett, M. R. Anver, M. Baseler, and D. L. Longo. 1992. Human-mouse lymphoid chimeras: host-vs.-graft and graft-vs.-host reactions. *Eur.J.Immunol.* 22:1421-1427.
187. Tournoy, K. G., S. Depraetere, R. A. Pauwels, and G. G. Leroux-Roels. 2000. Mouse strain and conditioning regimen determine survival and function of human leucocytes in immunodeficient mice. *Clin Exp Immunol* 119:231-239.
188. Lubin, I., H. Segal, H. Marcus, M. David, L. Kulova, M. Steinitz, ErlichP., J. Gan, Y. Reisner, H. Segall, Erlich, and P. 1994. Engraftment of human peripheral blood lymphocytes in normal strains of mice. *Blood* 83:2368-2381.
189. Murray, A. G., P. Petzelbauer, C. C. W. Hughes, J. Costa, P. Askenase, and J. S. Pober. 1994. Human T-cell-mediated destruction of allogenic dermal microvessels in a severe combined immunodeficient mouse. *Proc.Nat'l.Acad.Sci.USA* 91:9146-9150.
190. Murray, A. G., J. S. Schechner, D. E. Epperson, P. Sultan, J. M. McNiff, C. C. Hughes, M. I. Lorber, P. W. Askenase, and J. S. Pober. 1998. Dermal microvascular injury in the human peripheral blood lymphocyte reconstituted-severe combined immunodeficient (HuPBL-SCID) mouse/skin allograft model is

T cell mediated and inhibited by a combination of cyclosporine and rapamycin. *Am J Pathol.* 153:627-638.

191. Sultan, P., J. S. Schechner, J. M. McNiff, P. S. Hochman, C. C. Hughes, M. I. Lorber, P. W. Askenase, and J. S. Pober. 1997. Blockade of CD2-LFA-3 interactions protects human skin allografts in immunodeficient mouse/human chimeras. *Nature Biotechnology* 15:759-762.
192. Torbett, B. E., G. Picchio, and D. E. Mosier. 1991. HuPBL/SCID mice: a model for human immune function, AIDS, and lymphomagenesis. *Immunol.Rev.* 124:139-164.
193. King, M. Pearson T. Rossini A. A. Shultz L. D. and Greiner D. L. Humanized mice for the study of type 1 diabetes and beta cell function. New York Academy of Sciences . 2008.

Ref Type: In Press

194. Nervi, B., M. P. Rettig, J. K. Ritchey, H. L. Wang, G. Bauer, J. Walker, M. L. Bonyhadi, R. J. Berenson, J. L. Prior, D. Piwnica-Worms, J. A. Nolta, and J. F. DiPersio. 2007. Factors affecting human T cell engraftment, trafficking, and associated xenogeneic graft-vs-host disease in NOD/SCID beta2mnull mice. *Exp.Hematol.* 35:1823-1838.
195. Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K. R. Cooke, and J. L. Ferrara. 2002. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat.Med.* 8:575-581.
196. Brown, G. R., E. L. Lee, and D. L. Thiele. 2003. TNF enhances $CD4^+$ T cell alloproliferation, IFN-gamma responses, and intestinal graft-versus-host disease by IL-12-independent mechanisms. *J.Immunol.* 170:5082-5088.
197. Cooke, K. R., G. R. Hill, J. M. Crawford, D. Bungard, Y. S. Brinson, J. Delmonte, Jr., and J. L. Ferrara. 1998. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J.Clin.Invest* 102:1882-1891.
198. Hattori, K., T. Hirano, H. Miyajima, N. Yamakawa, M. Tateno, K. Oshimi, N. Kayagaki, H. Yagita, and K. Okumura. 1998. Differential effects of anti-Fas ligand and anti-tumor necrosis factor alpha antibodies on acute graft-versus-host disease pathologies. *Blood* 91:4051-4055.
199. Gilliam, A. C., D. Whitaker-Menezes, R. Korngold, and G. F. Murphy. 1996. Apoptosis is the predominant form of epithelial target cell injury in acute experimental graft-versus-host disease. *J.Invest Dermatol.* 107:377-383.
200. Piguet, P. F., G. E. Grau, B. Allet, and P. Vassalli. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J.Exp.Med.* 166:1280-1289.
201. Remberger, M., O. Ringden, and L. Markling. 1995. TNF alpha levels are increased during bone marrow transplantation conditioning in patients who develop acute GVHD. *Bone Marrow Transplant* 15:99-104.
202. Holler, E., H. J. Kolb, A. Moller, J. Kempeni, S. Liesenfeld, H. Pechumer, W. Lehmacher, G. Ruckdeschel, B. Gleixner, C. Riedner, and . 1990. Increased

serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. *Blood* 75:1011-1016.

203. Holler, E., H. J. Kolb, R. Hintermeier-Knabe, J. Mittermuller, S. Thierfelder, M. Kaul, and W. Wilmanns. 1993. Role of tumor necrosis factor alpha in acute graft-versus-host disease and complications following allogeneic bone marrow transplantation. *Transplant Proc.* 25:1234-1236.
204. Levine, J. E., S. Paczesny, S. Mineishi, T. Braun, S. W. Choi, R. J. Hutchinson, D. Jones, Y. Khaled, C. L. Kitko, D. Bickley, O. Krijanovski, P. Reddy, G. Yanik, and J. L. Ferrara. 2008. Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. *Blood* 111:2470-2475.
205. Gattinoni, L., S. E. Finkelstein, C. A. Klebanoff, P. A. Antony, D. C. Palmer, P. J. Spiess, L. N. Hwang, Z. Yu, C. Wrzesinski, D. M. Heimann, C. D. Surh, S. A. Rosenberg, and N. P. Restifo. 2005. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J.Exp.Med.* 202:907-912.
206. Prlic, M. and S. C. Jameson. 2002. Homeostatic expansion versus antigen-driven proliferation: common ends by different means? *Microbes.Infect.* 4:531-537.
207. Goldrath, A. W., C. J. Luckey, R. Park, C. Benoist, and D. Mathis. 2004. The molecular program induced in T cells undergoing homeostatic proliferation. *Proc.Natl.Acad.Sci.U.S.A* 101:16885-16890.
208. Marleau, A. M. and N. Sarvetnick. 2005. T cell homeostasis in tolerance and immunity. *J.Leukoc.Biol.* 78:575-584.
209. Surh, C. D. and J. Sprent. 2000. Homeostatic T Cell Proliferation. How far can t cells be activated to self-ligands? *J.Exp.Med.* 192:F9-F14.
210. Murali-Krishna, K. and R. Ahmed. 2000. Cutting edge: naive T cells masquerading as memory cells. *J.Immunol.* 165:1733-1737.